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Full paper

Enzyme-Polymer Conjugates to enhance enzyme shelf life in a liquid detergent formulation[a]

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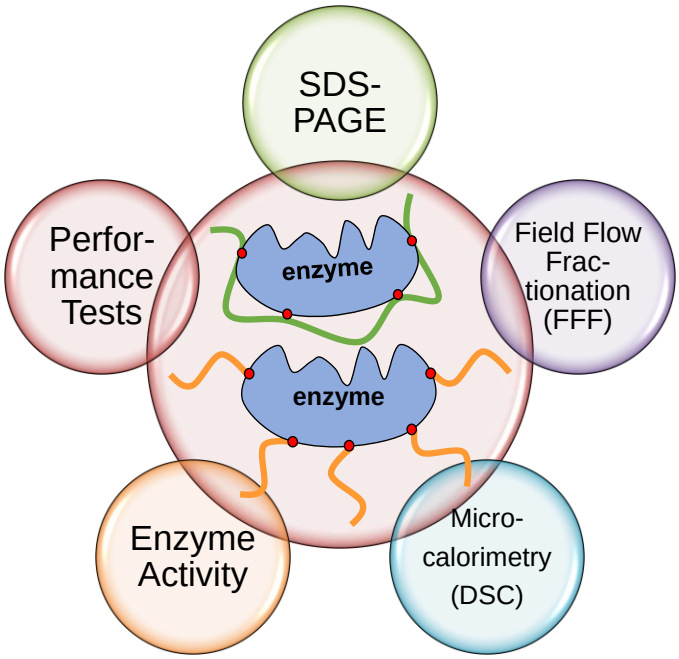
Abstract

Herein, we report the synthesis of enzyme-polymer conjugates. Four different activated polymers (mPEG-aldehyde, mPEG-NHS, maltodextrin-aldehyde, carboxymethyl cellulose aldehyde) are conjugated to the surface of protease, α-amylase and lipase using two different strategies (reductive amination and alkylation with NHS activated acid). Although the chemical modification of the enzymes is accompanied by slight losses in enzyme activity (maximum loss 40%), the covalent attachment of polymers increased the thermal stability and the stability in a standard detergent formulation compared to the unmodified enzymes. The enzyme-polymer conjugates are characterized by asymmetrical-flow-field-flow fractionation and differential scanning

[a] **Supporting Information** is available online from the Wiley Online Library or from the author.

microcalorimetry. Furthermore, we demonstrate that conjugated enzymes still show performance in a real washing process. Enzyme-polymer conjugates show a potential as a stabilizing system for enzymes in detergents.

Figure for ToC



1. Introduction

Enzymes are highly specific regarding their substrate and they represent as an environmentally friendly bio-catalysts. With initial research, carried out during the DNA revolution of the 1980s, applications for enzymes in industrial applications are now reality in several sectors such as pharmaceutical, food or detergent industry.[1],[2] For a clinical application, many proteins lack in solubility and stability. *In vivo* this results in hydrolysis, degradation and a loss of function. Other disadvantages include short half-life in organisms and the potential of an immunogen effect.[3] Not only is the application of enzymes as a drug limited, but also other applications since enzymes are

sensitive to non-physiological conditions such as extreme pH values, high temperatures or detergents. One opportunity to overcome these problems is enzyme encapsulation.

Polymeric nanocarriers[4],[5],[6],[7], micelles[8],[9], liposomes[10],[11], particles[12] and capsules[13],[14],[15] belong to these aforementioned enzyme stabilization systems. Another strategy for enzyme stabilization is the covalent coupling of an enzyme to an inert, biocompatible, water soluble polymer. For this conjugation reaction, various polymers have been investigated. These include polyethylene glycol (PEG), hydroxyethyl starch (HES)[16], dextrane[17], hyaluronic acid[18], polyvinylpyrolidon (PVP)[19], polyvinylalcohol (PVA)[20], polyethylenimine (PEI)[21] and polyphosphate[22]. The stabilizing effect of such polymers on enzymes can be attributed especially to a shielding and protection of the enzyme against denaturing conditions. PEG an create a water shell around the enzyme which enables solubility and activity in polar organic solvents.[23] Furthermore the steric hindrance of PEG can prevent dissociation and autolysis of an enzyme.[24]

By far, the most common used polymer is the Food and Drug Administration (FDA) approved PEG.[25] The polymers are functionalized with a group which is reactive towards one of the enzyme amino acids. Predominantly the polymers bear an electrophilic group which reacts with a nucleophilic amino acid, such as cysteine, lysine, arginine, serine, threonine or tyrosine.[26] Under physiological conditions enzyme and polymer form a covalent linkage and an enzyme-polymer conjugate is formed. Such enzyme-polymer conjugates can improve the potential of enzymes for industrial applications. This is on their stability enhancement e.g. polymer conjugated enzymes are better protected against enzymatic degradation and consequently the *in vivo* halftime is higher compared to the native enzyme.[27] These advantages result in an increasing number of approved pharmaceutics based on an enzyme-polymer conjugate – started with Adagen® in 1990.[25] The immobilization of enzymes on nanoparticles represents another promising strategy for enzyme

conjugates. Apart from the benefits in enhanced enzyme activity and stability, new vistas in biocatalysis are open up.[28],[29],[30],[31],[32]

Although, investigated a lot in the context of biomedicine and drug delivery, enzyme-polymer conjugated are not considered in the context of detergent applications. A detergent represents an environment which initiates enzyme denaturation. This is due to chelating agents destabilizing calcium depending enzymes, linear alkylbenzenesulfonates starting the unfolding of enzymes and, in addition, proteases decompose themselves and other enzymes. Therefore, the long-term stabilization of enzymes in standard detergent formulations is a particular challenge. A real and lasting solution to this challenge is, to the best of our knowledge, non-existing.

Yang *et al.* showed that the proteolytic degradation of proteases (subtilisins) regarding conjugated enzymes – subtilisin as well – is decreased and the conjugates had at least three times higher life-time compared to the native enzyme.[33] Measuring the enzyme activity of an enzyme-polymer conjugate in an SDS solution (0.3% (w/v)), Villalonga *et al.* showed that the conjugation reaction increases enzyme stability against denaturing surfactants.[34] The covalent attachment of PEG chains increases the stiffness and decreases the flexibility which leads to an improved enzyme stability. Furthermore, Minten *et al.* describe the formation of a shield formed by water bonding around the enzyme which protects the enzyme sterically.[2] Schroeder *et al.* covalently attached PEG to a protease and could increase the half-life of the enzyme in a commercial detergent solution by nearly a factor of two.[35] In a similar manner, a covalently attached polysaccharide can stabilize an enzyme by preventing unfolding. With polysaccharides it is possible to get a multipoint attachment, if there is more than one reactive functional group per polymer molecule.[34, 36] Nevertheless, some challenges still exist with respect to modifying an enzyme covalently. In many cases enzyme conjugation correlates with a loss of activity. Wich *et al.* observed a remaining

enzyme activity of between 13 and 80% in lysozyme conjugated with four different activated polyethylene glycols.[37]

In this study we investigated the conjugation of enzymes with polymers aiming for a long-term stabilization of the enzyme in a standard detergent formulation. Therefore, three different laundry-relevant enzymes (protease, α-amylase and lipase) are covalently linked to four different functional polymers using their amino groups (lysine residue or *N*-terminus) for conjugation. The entire process starting with polymer functionalization, identifying lead candidates and finally characterising and testing those candidates is systematically investigated. The conversion rate is determined by SDS-PAGE, analytical ultracentrifugation and HPLC. After purification and isolation, the conjugated enzymes by dialysis, the enzyme concentration and activity is measured. The work continues with lead candidates which show a conversion yield of 100%. The selected optimal enzyme-polymer conjugates are further characterized regarding molar mass, thermal stability and stability in detergents. Finally, results of an application-oriented performance test of enzyme-polymer conjugates are discussed.

In brief, by these straightforward reactions a complete conjugation with all three enzymes without a substantial decrease in enzyme activity for the lead candidates has been achieved. Enzyme-polymer conjugates allow short-term stabilization (up to four weeks) of the enzymes against a standard detergent formulation while maintaining their washing performance. Furthermore, the thermal stability of the enzymes is enhanced.

2. Experimental Section

2.1 Materials and methods

All chemicals and materials were used without further purification. Demineralized water was used for all experiments. The pH values of the buffer solutions were adjusted using concentrated hydrochloric acid (Bernd Kraft GmbH, Germany) and sodium hydroxide solutions (Sigma-Aldrich Corperation, USA). The three enzymes used Savinase® 16 L (*Bacillus subtilis*, liquid), α-amylase (*Bacillus licheniformis*, lyophilized powder) and Lipolase® 100L (*Thermomyces lanuginosus*, liquid) were purchased from Sigma-Aldrich. The polymers used mPEG-propionaldehyde (5 kDa) and mPEG-succinimidyl succinate ester were obtained from Sigma-Aldrich and from Biochempeg Scientific Inc. (USA) respectively. The polysaccharide carboxymethyl cellulose was purchased from CP Kelco (USA), maltodextrin from Sigma-Aldrich. α-Glucosidase (multifunctional) and ethylidene-blocked 4-nitrophenylmaltoheptaoside (EPS) were obtained from Roche Applied Science (Germany). Suc-Ala-Ala-Pro-Phe-*para*-Nitroanilin (AAPF) was purchased from Bachem Holding AG (Switzerland) and 4-Nitrophenyl valerate from Sigma-Aldrich. Sodium phosphate dibasic, Sodium cyanoborohydride, Trizma base, Brij® L23, gum arabica, Sodium acetate trihydrate, 3-(*N*-morpholino)propanesulfonic acid (MOPS), Tween®20, bicine and trichloroacetic acid were purchased from Sigma-Aldrich. Sodium chloride was obtained from Bernd Kraft GmbH, polyethylene glycol (6 kDa) from Merck Millipore (USA) and Texapon® N70 as well as the standard detergent formulation ES1 from BASF SE (Germany).

Oxidation and functionalisation of the polysaccharides used was performed according to Yi *et al.*.[38] Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to well-known protocols.[39] In brief, a discontinuous polyacrylamide gel (NuPAGE 4-12% Bis-Tris Protein Gels, 1 mm; Thermo Fisher Scientific) with a stacking (4% acrylamide) and a separating gel (12% acrylamide) was used. See Blue™ plus 2 (Thermo Fisher Scientific) was used as the marker and the gel is stained with Coomassie Brilliant Blue R (Sigma-Aldrich). Samples with protease were precipitated with a solution of 50% trichloroacetic acid and washed

with acetone.[40] Measurements with high-performance liquid chromatography were performed with a 1260 Infinity (Agilent Technologies, USA), equipped with a reversed phase column and a DAD detector.

2.2 Enzyme Assays

Enzyme concentrations were determined by measuring UV absorption at 280 nm and by performing a bicinchoninoic acid assay using a Pierce BCA Protein Assay Kit (Thermo Scientific, USA).[41] The assays for determination of enzyme activity were consisting of tailored substrates and were specific to one enzyme. Laboratory instructions were taken from literature: protease,[42] and α-amylase[15] [43]. The lipase assay was adapted from Palacios *et al.*.[44]

2.3 Differential Scanning Calorimetry

Differential scanning calorimetry measurements regarding thermal stability were carried out using a nanoDSC (TA Instruments, USA). All samples were adjusted to an enzyme concentration of 2 mg/mL (100 mM phosphate buffer, pH=9) and were degassed under vacuum for 25 minutes before being loaded into the autosampler. A scan rate of 1.5 K/min in a temperature range of 20 to 110 °C was utilized. For analysis, the software package nanoAnalyze (Version 3.4.0, TA Instruments) was used. Measured thermograms were baseline corrected and the T_{max} value was determined.

For this purpose, enzymes with covalently linked polymers and native enzymes in presence of polymers went through a temperature program of the nanoDSC. With the reference measurement, it was examined if the presence of a polymer alone is sufficient enough to increase the thermal stability of an enzyme. The ratio between polymer and enzyme was the same in reference and conjugate measurements. For conformational stability, the T_{max} value was determined. The

temperature T_{max} was located at the peak maximum of the transition from folded to unfolded enzyme state.

2.4 Modification of Enzymes

2.4.1 Modification of Enzymes with Polysaccharide-Aldehydes

The enzyme was dissolved in phosphate buffer (100 mM, pH=9) and the appropriate polysaccharide-aldehyde (CMC, maltodextrin) was added. The polymer was added in a molar excess of 50 with respect to the enzyme. After two hours of stirring at 22 °C, sodium cyanoborohydride was added with a tenfold molar excess with respect to the polymer. Stirring continued for about three days at room temperature. The enzyme-conjugate was then purified by dialysis (SnakeSkin™ Dialysis Tubing 10K MWCO, Thermo Fisher) against water for 24 hours and was then lyophilized.

2.4.2 Modification of Enzymes with mPEG-Aldehyde

Enzyme was dissolved in phosphate buffer (100 mM, pH=9) and mPEG-aldehyde added in a molar excess of 50 with respect to the enzyme. After two hours of stirring at 22 °C, sodium cyanoborohydride was added with a tenfold molar excess to the polymer. Stirring continues for 24 hours at room temperature. The enzyme-conjugate was purified by dialysis (SnakeSkin™ Dialysis Tubing 10K MWCO, Thermo Fisher) against water for 24 hours and was then lyophilized.

2.4.3 Modification of Enzymes with mPEG-NHS

Enzyme was dissolved in phosphate buffer (100 mM, pH=9) and mPEG-NHS as added to the solution. The polymer had a molar excess of about 50 in respect to the enzyme. The mixture was stirred at 22 °C for about 24 hour. The enzyme-conjugate was then purified by dialysis

(SnakeSkin™ Dialysis Tubing 10K MWCO, Thermo Fisher) against water for 24 hours and was then lyophilized.

2.5 Asymmetrical-Flow Field-Flow Fractionation (AF4)

The AF4 experiments were performed using the Eclipse AF4 separation system from Wyatt Technology combined with a multidetection system (UV absorption at 280 nm, multiangle light-scattering (MALS), quasi-elastic light-scattering (QELS) and differential refractive index (DRI)). The enzyme-polymer conjugates, with an enzyme concentration of 2 to 4 mg/mL were injected into the trapezoidal long channel LC equipped with a 10 kDa RC-membrane (Regenerated Cellulose, Millipore). The separations were carried out at room temperature. The running eluent prepared with ultrapure water (MilliQ, 18Ω) water was a 4 mM tris buffer (Thermo Fisher, Corporation, USA). A focusing/injection step of 3 min with a focusing-flow rate of 3 mL/min and an injection-flow rate of 0.2 mL/min was followed by a focusing/relaxation step of an additional 1 min. The sample volume injected into the channel was 20 µL. An elution step was performed with a constant cross-flow for 15 min at 3 mL/min followed by an exponentially decreasing cross-flow rate, from 3 to 0.1 mL/min in 10 min. The detector-flow rate was 0.5 mL/min.

Data acquisition and evaluation was performed using the Astra 6.1.5.22 software (Wyatt Technology Corporation, USA). The determination of M_W values from MALS as achieved using the specific refractive index increment of dn/dc=0.15 for scattering angles from 37.5° to 140° (detectors 5–16) and data treatment according to Zimm formalism using a first order exponential for data fitting. In order to have an exclusive enzyme detection, absorption at a wavelength of 280 nm was used.

2.6 Storage Tests

Before addition of pure or conjugated enzyme to the standard detergent formulation, the pH value was corrected to 8.5 using aqueous potassium hydroxide. After enzyme addition, the solutions were stored at 25°C and the enzyme activity was measured at different dates for four weeks.

2.7 Washing and Performance Tests

For washing and performance tests 200 mL of a standard detergent formulation which was part of a commercial brand was mixed with 48 mL hard water (sodium hydrogen carbonate, magnesium chloride and calcium chloride; water hardness 2.5 mM). 2 mL enzyme solution (0.1, 0.5, 0.7 ppm) were added, before the solution was filled in a washing pot together with respectively 10 g cotton (4x 2.5 g BW 283) and 10 g dirty cloths (2x 2.5 g EMPA 161 maize starch, 2x 2.5 g CFT-C-S-28 rice starch) and 20 steel balls. The washing pot was transferred into a Lander-O-meter (AATCC, USA) and the washing process started. The washing cycle was performed at 25 and 40 °C in each case for 30 minutes. L* value of Lab colour space was measured before and after washing using a reflection photometer.

3. Results and discussion

3.1 Synthesis and characterization of conjugates

In order to synthesize enzyme-polymer conjugates, three different laundry-relevant enzymes have been mixed with four different activated polymers (**figure 1**). The enzyme-polymer coupling were performed with a high excess of polymer to give a polymer density which was as high as possible. The polymers carry functional groups reactive towards covalent coupling with amino groups, which are present as lysine residues or *N*-terminus at the enzymes. All modifications were performed in buffered aqueous solutions under physiological conditions. Amongst the polymers

tested two monofunctional PEGs (mPEG-aldehyde and mPEG-NHS) and two multifunctional polysaccharides (maltodextrin- and CMC-aldehyde) showed promising results. Covalent attachment of the polysaccharides can result in a multipoint attachment, which means that one polymer can have more than one connection point to one enzyme. In the case of the PEGs, only a single point attachment (figure 1) is possible due to one reactive group per molecule polymer. These polymers were selected due the various attachment types.

PLEASE INSERT FIGURE 1 HERE.

Via SDS-PAGE and HPLC the conversion rates were analysed (SDS-PAGE is shown in SI). Determining the conversion rate, it is crucial that no unmodified enzyme is left. This means that at least every enzyme molecule is conjugated to at least one polymer chain. Conjugation of α-amylase and lipase occurred very efficiently with conversion rates of 100% for all polymers (**table 1**). In contrast to these results, protease, only showed a complete conversion with polysaccharides. With activated PEGs only a conversion of 45% could be observesd (table 1). This is most probably due to the lower number of accessible amino groups on the protease surface (3 Lys) compared to the surface of α-amylase (8 Lys) or lipase (7 Lys), as determined using computer simulations. The conjugation reaction was completed, when no native enzyme was left or after a maximum of three days.

The reaction solutions were purified by dialysis to remove reducing agent and non-attached polymer. Purified enzyme-polymer conjugates were freeze-dried, before measuring the content of enzyme. Enzyme concentration of the conjugates was determined using a BCA assay as well as the measurement of absorption at 280 nm (data not shown). Based on these results, enzyme specific assays were used to determine the activity of the conjugates. Like this, the loss of enzyme activity

caused by conjugation was calculated (table 1). Protease conjugates with mPEG-aldehyde and maltodextrin aldehyde retained a maximum of enzyme activity (92 and 94%). Lipase and α-amylase kept about 80 to 90% of their initial activity. For all three enzymes, the enzyme activity was generally lowered if CMC-aldehyde was used (table 1)

Molar mass of the enzyme-polymer conjugates was measured using AF4 (table 1). Molar masses of 28 kDa for protease (unmodified), 67 kDa for α-amylase (unmodified) and 40 kDa for lipase (unmodified) were obtained. The activated PEGs had a molar mass of 5 kDa, maltodextrin aldehyde 20 kDa and CMC-aldehyde 10 kDa. Comparing the molar mass before and after enzyme-polymer conjugate formation (table 1) protease carried one covalently linked polymer chain in its polymer conjugate, whereas lipase and α-amylase were linked to up to three or eight polymer chains, respectively (table 1). Consequently, at least half of the assessible amino groups of all three enzymes were conjugated with a polymer whilst the other half remained unmodified.

PLEASE INSERT TABLE 1 HERE.

For further characterisation, the thermal stability of the synthesized enzyme-polymer conjugates was determined using differential scanning microcalorimetry (nanoDSC); increasing higher T_{max} values indicate increasing enzyme stability. A shift of T_{max} for references and conjugates as compared to the native enzyme under identical buffer conditions was observed (**figure 2**). Thereby, a higher T_{max} for a conjugated enzyme as compared to the unmodified enzyme was illustrated by a positive bar and represents an increased thermal stability of the enzyme. Lower T_{max} values represented by a negative bar show a reduced thermal stability. The absolute measured T_{max} value is shown as a number next to the bars in figure 2 and additionally listed in table 1 The original data of recorded thermograms are given in the SI.

Under the present conditions α-amylase showed a T_{max} value of 86 °C. This value was slightly increased after α-amylase conjugation to 88 °C for mPEG-aldehyde and to 90 °C for the CMC-aldehyde. Conjugated with mPEG-NHS (95 °C) or maltodextrin-aldehyde (93 °C) the thermal stability of α-amylase was increased even more significantly. The presence of polymer without enzyme conjugation reduced enzyme thermal stability in all cases and especially in case of CMC conjugation (82 °C; figure 2). This further proved the positive effect of enzyme-polymer conjugate formation on the enzyme thermal stability.

Protease showed a T_{max} value of 67 °C. A substantial stabilization occurred if protease was conjugated with maltodextrin-aldehyde. In that case the thermogram showed a bimodal shape with the main peak located at 81 °C. All other conjugations investigated as well as the presence of polymers without conjugation had no detectable influence on the thermal stability of protease (figure 2). Interestingly, lipase behaved similarly to protease with a T_{max} value at 68 °C and an altered thermal stability after conjugation to mPEG-NHS (T_{max} 75 °C) or maltodextrin-aldehyde (T_{max} 73 °C). All other conditions investigated did not show a detectable influence on the lipase´s thermal stability (figure 2).

In addition, the presence of polymers without covalent conjugation to the enzymes showed no positive effect and in case of α-amylase even a negative influence on enzyme stability. In the case of PEG conjugation this effect resulted from reducing polarity, favouring unfolding of enzymes and resulting in a reduced T_{max} value.[45] Positive effects regarding thermal stability increased caused by polymer conjugation could be attributed to an enhanced rigidity of the enzyme. A more rigid enzyme showed a reduced structural dynamics which protects against conformational changes and temperature induced unfolding.[46] In addition the thermal stability is increased by a shielding effect of the PEG chain which protects the enzyme sterically against autolysis and prevents dissociation of the enzyme.[24]

Absent effects can derive from a compensation of increased rigidity and a pronounced hydrophobic interaction. This interaction can occur between PEG and hydrophobic amino acids and initiate an unfolding of the enzyme.[45] However, it is considered that the thermal stability of an enzyme-polymer conjugate is independent of the polymers molar mass and dependent mainly upon the number of attached polymer chains. In this case, the T_{max} value should increase until a saturation value.[46] To reach this saturation, as many amino groups as possible needed to be conjugated to polymers. The specific local surrounding of the polymer on the enzyme´s surface, and the location of polymer conjugation have a particular impact on its thermal stability.[47] This had been observed for α-amylase and mPEG-aldehyde and even more pronounced for mPEG-NHS-α-amylase conjugates. Here, the conjugation taking place over the active ester led to a significant increase in the enzyme thermal stability. This had not been observed for α-amylase conjugated to mPEG-aldehyde. Interestingly, covalent attachment of maltodextrin-aldehyde increased the thermal stability of all tested enzymes. This stabilising effect might result from the possible multipoint-attachment of maltodextrin-aldehyde to each enzyme. This resulted in a conservation of the native and active structure of the enzyme and the denaturation had been prevented. It is worth noting that in the case of protease-polymer conjugates the peaks were narrower as compared to the pure enzyme. For mPEG-aldehyde-protease conjugates the T_{max} value was identical to the unmodified enzyme but the onset temperature at which the denaturation of the main peak starts was shifted to higher temperatures. The onset temperature of protease-mPEG-aldehyde conjugate was increased by 8 K (see SI). This strongly indicates a protection of conjugated protease against proteolysis.

PLEASE INSERT FIGURE 2 HERE.

3.2 Storage tests with conjugates

Storage tests were performed with the unmodified enzymes and enzyme-polymer conjugates in a standard detergent formulation at room temperature for defined time intervals. The enzyme activity was measured regularly to evaluate the conjugated enzyme stability in comparison to the unmodified enzyme. The conditions in a liquid detergent formulation are extremely harsh for enzymes as a wide range of different surfactants is present. The formulation used includes two anionic and one non-ionic surfactant. Anionic surfactants initiate the thermodynamically favoured unfolding of enzymes.[48] The damaging effect is particularly present for protease and amylase.[49] In addition, protease shows (auto)-proteolytic properties and as a result this enzyme can degrade itself or other enzymes.[42]

Reference samples containing non-covalently bound polymers showed that the presence of polymers without any covalent attachment to the enzyme did not have a positive influence on the enzyme activity and stability within the formulation. The subsequent storage test had been performed with protease conjugated to maltodextrin- and CMC-aldehyde and with all tested conjugated of α-amylase and lipase because all of them were showing 100% conversion to the enzyme-polymer conjugate (table 1). Time-dependent enzyme activities of unmodified and polymer-conjugated enzymes are shown in **figure 3**. All four α-amylase-polymer-conjugates showed improved stability up to three weeks. The strongest stabilization effect was observed for α-amylase CMC-aldehyde conjugates (figure 3). Conjugated to the anionic polysaccharide more than 40% of the α-amylase was still active after two weeks, whereas the unmodified α-amylase did not shown any activity any more after this time. After four weeks, α-amylase conjugates did almost not show enzyme activity any more (figure. 3). Protease was more sensitive against the detergent formulation as compared to α-amylase. Already after one week no activity is detected any more for the unmodified enzyme. Protease covalently linked to CMC-aldehyde was denatured even

faster than the native enzyme. However, protease conjugated to maltodextrin showed a noticeable higher stability in the formulation, with 20% still being active after four weeks (figure. 3). Interestingly, lipase was significantly more stable in detergents than both of the other enzymes. After four weeks 60% of the unmodified lipase was still active. This stability was reduced if lipase was conjugated to polysaccharides such as maltodextrin- and CMC-aldehyde for which no lipase activity can be measured after four weeks. Nevertheless, lipase stability was increased using PEG-conjugates. Lipase covalently linked to mPEG-NHS maintained its activity after four weeks at almost 90% (figure. 3).

Stabilizing effects observed for α-amylase-CMC-aldehyde, protease-maltdextrin-aldehyde and lipase-mPEG-NHS resulted from an increased rigidity of the enzymes covalently linked to polymer chains, similarly to the thermal stability increase. The shielding caused by hydrogen bonds to the polymer around the enzymes also gave a stabilizing effect.[2, 46] Due to the conjugation to lysine residues the number of positive groups on the proteins surface which can interact with negatively charged surfactants was reduced. It should also be noted that stabilizing and destabilizing polymers strongly depend on the different enzyme. CMC-aldehyde for example significantly stabilized α-amylase and but decreased stability of protease and lipase (figure 3). In summary, all three enzymes could be stabilized in a detergent formulation over short-term of four weeks using conjugation to a polymer.

PLEASE INSERT FIGURE 3 HERE.

3.3 Performance tests with conjugates

Once having proven that the enzyme stability in a detergent formulation by the covalent attachment of polymers on the enzyme surface, two selected α-amylase-conjugates were evaluated in a

performance test. In these experiments the performance of α-amylase in removing stains had been analysed. The aim had been to achieve the same or better washing performance for conjugated enzymes as for unmodified enzymes. These experiments are especially necessary, to prove enzyme activity on a large scale and for technically relevant applications. Therefore, it has to be ruled out that the active site of the enzyme is blocked and thus inasseccible for real stain removal after attachment of polymer chains onto the enzyme surface. In these performance tests, three different concentrations of pure α-amylase and α-amylase-maltodextrin- and mPEG-aldehyde conjugates had been washed at 25 and 40 °C together with dirty cloths. The cloths, which have maize and rice starch stains, were analysed regarding their L* value using a photometer. Comparing photometer measurements before and after the washing process allows the quantification of the washing success which is related to enzyme performance. The resulting dL* value was plotted against the enzyme concentration (ppm). From the results presented in **figure 4** it is evident that the maltodextrin conjugate of α-amylase performed as well as the unmodified α-amylase. On the other hand, mPEG-aldehyde-α-amylase conjugate showed a performance decreased of almost down to 50% of the original unmodified enzyme performance (figure 4). This might be due to a shielded active site of the enzyme thereby reducing its efficiency in removing stains. On the basis of these results, enzymes with a covalent multi-attachment of polysaccharides were more favourable in a detergent application than single-attached PEG polymers.

PLEASE INSERT FIGURE 4 HERE.

4. Conclusion

In summary, the synthesis, characterization and washing performance of enzyme-polymer conjugates was reported. Three different laundry-relevant enzymes, protease, α-amylase and lipase

had been conjugated to different polymers and the attachment of polymer chains in a sufficient number on the surface of all three of these enzymes was demonstrated. Despite the chemical modification, the conjugated enzymes show a remaining activity of at least 60%, in most cases even significantly higher. Fractionating techniques are used to characterize the enzyme-polymer conjugates and to determine the molar mass of the conjugates revealing a minimum of two conjugated polymer chains per enzyme molecule. The thermal stability of the enzymes and the conjugates had been characterized via differential scanning microcalorimetry. Conjugates with maltodextrin-aldehyde could especially improve the thermal stability of the enzymes. The synthesized conjugates were able to stabilize the three enzymes against denaturation initiated by surfactants over short-term periods of one month. Finally, a performance washing test showed that α-amylase-maltodextrin-aldehyde conjugates were confirmed as a lead candidate for a laundry application showing the same washing performance as the unmodified enzyme.

In general, the synthesis of enzyme-polymer conjugates represents an effective method for stabilizing enzymes against temperature and detergents up to several weeks. In subsequent experiments, it will be attempted to expand this effect to a long-term application.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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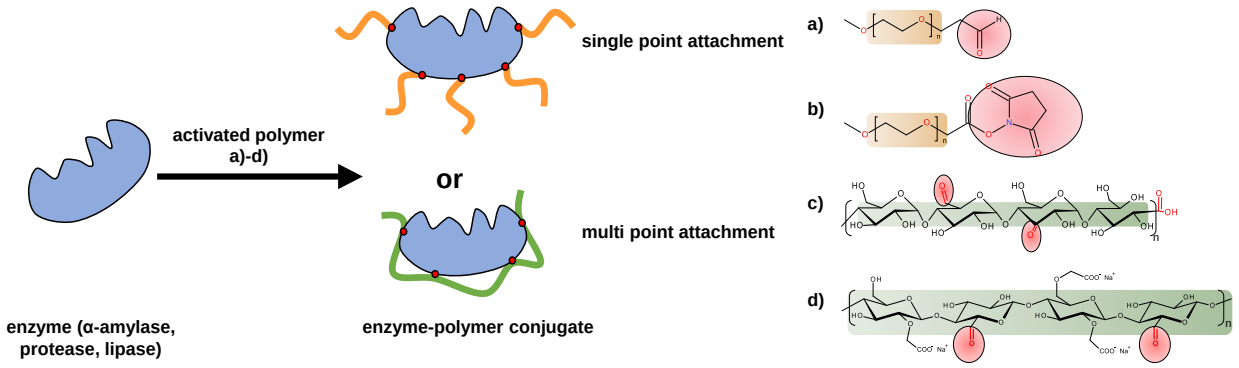

Figure 1: Enzyme modification with four different polymers: mPEG-aldehyde (a), mPEG-N-hydroxysuccinimide ester (b), maltodextrine aldehyde (c) and carboxymethyl cellulose aldehyde (d). The red circles indicate the functional group reactive towards covalent coupling with the enzyme´s amino group.

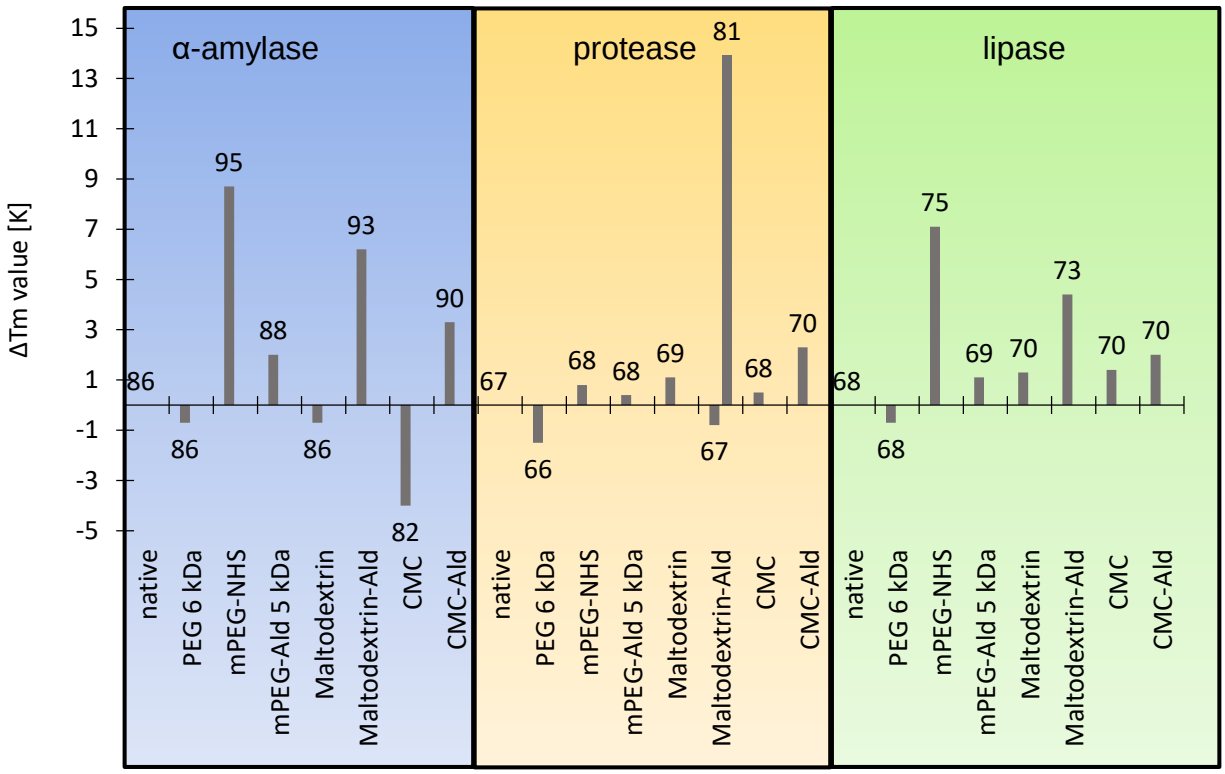

Figure 2: Overview of the results from the nanoDSC experiments of the pure enzyme alone and in presence of non-covalently linked polymer as well as the enzyme-polymer conjugates.

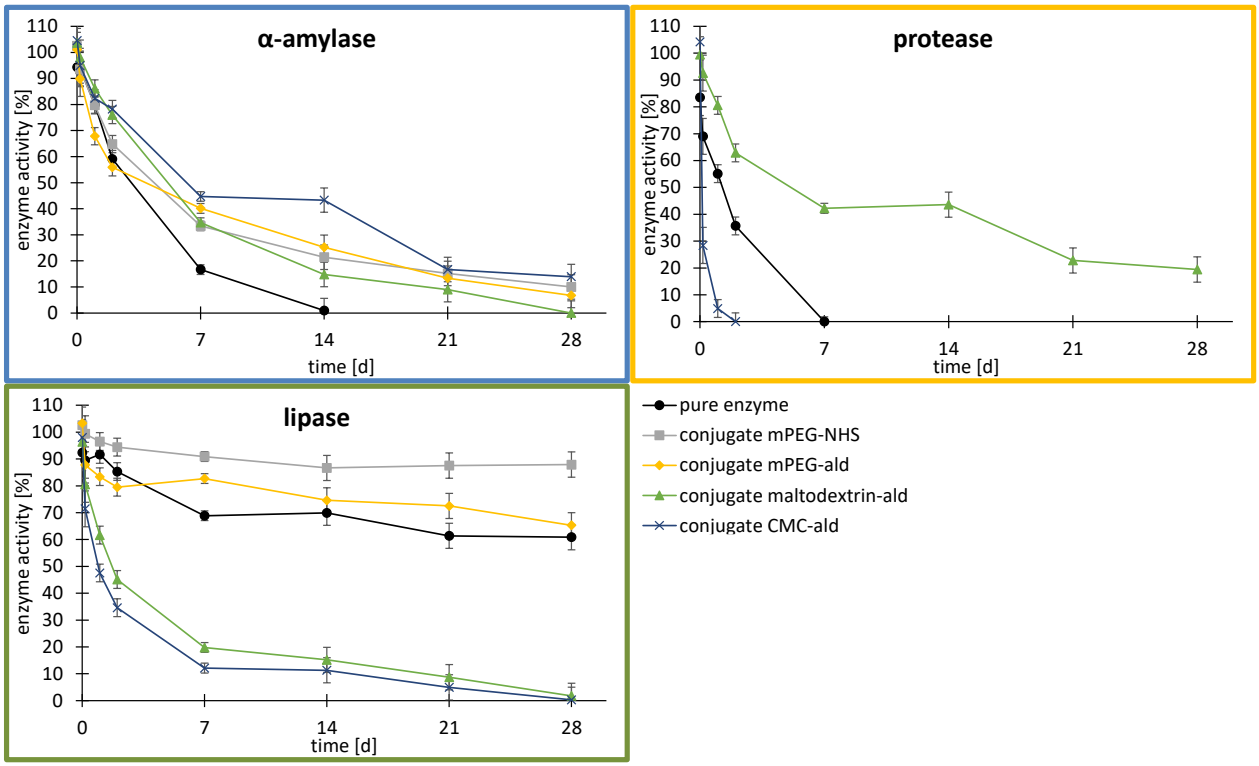

Figure 3: Graphical representation of the results of the storage test of the enzyme-polymer conjugates in a standard detergent formulation at room temperature for four weeks.

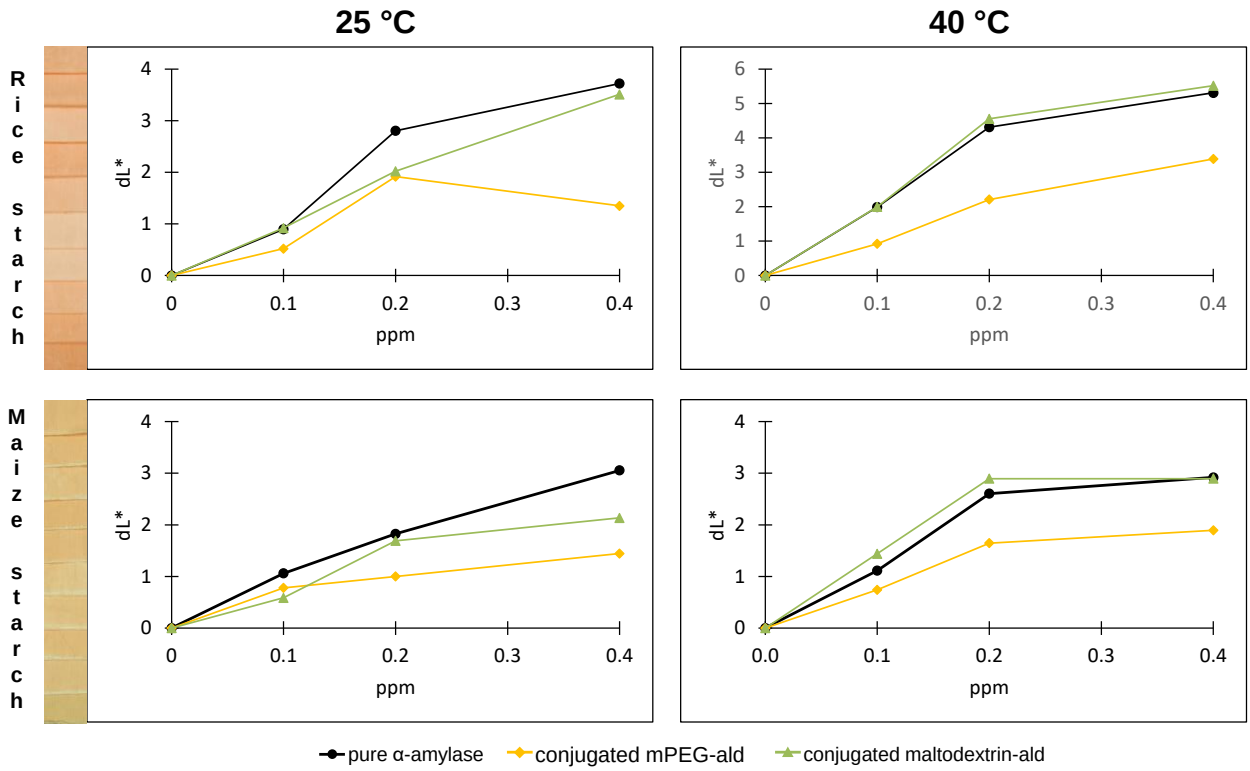

Figure 4: Results of the performance washing experiments of pure α-amylase and two conjugates (maltodextrin and mPEG-aldehyde). Both upper diagrams show the results with rice starch and the two lower diagrams with maize starch. Washing tests are performed at 25 and 40 °C.

Table 1: Resulting enzyme-polymer conjugate properties: conversion to conjugate, remaining enzyme activity, molar mass, number of attached polymer chains and T_{max} value for thermal stability.

Enzyme	**Polymer**	**Conversion [%][a)]**	**Enzyme Activity after Conjugation [%][b)]**	**Average molar mass [kDa]**	**# polymer chains**	**T_{max} value [°C]**
Protease	mPEG-aldehyde	45	92(±11)	36	1-2	67
	mPEG-NHS ester	44	60(±5)	Almost no conjugates detected	0	68
	Maltodextrin aldehyde	100	84(±10)	35	0-1	67 / 84

	CMC aldehyde	100	63(±13)	35	1	70
α-Amylase	mPEG-aldehyde	100	83(±11)	80	3	88
	mPEG-NHS ester	100	94(±6)	107	8	95
	Maltodextrin aldehyde	100	78(±11)	79	1	93
	CMC aldehyde	100	81(±12)	105	4	90
Lipase	mPEG-aldehyde	100	93(±2)	48	1-2	69
	mPEG-NHS ester	100	89(±7)	56	3	74
	Maltodextrin aldehyde	100	73(±4)	43	0-1	73
	CMC aldehyde	100	59(±11)	53	1	70

[a)] 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.

[b)] The enzyme activity after conjugation refers to the BCA and UV assay determined total enzyme concentration. The percentage of enzyme that remained active despite conjugation is stated.

Short Summary

We report the synthesis of enzyme polymer conjugates. Four different activated polymers are conjugated to the surface of protease, α amylase and lipase using two different strategies. Although the chemical modification of the enzymes is accompanied by slight losses in enzyme activity, the covalent attachment of polymers increased the thermal stability and the stability in a standard detergent formulation compared to the unmodified enzymes.