Polymer Degradation Hot Paper

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Synthesis of Modified Poly(vinyl Alcohol)s and Their Degradation Using an Enzymatic Cascade

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Abstract: Poly(vinyl alcohol) (PVA) is a water-soluble synthetic vinyl polymer with remarkable physical properties including thermostability and viscosity. Its biodegradability, however, is low even though a large amount of PVA is released into the environment. Established physical-chemical degradation methods for PVA have several disadvantages such as high price, low efficiency, and secondary pollution. Biodegradation of PVA by microorganisms is slow and frequently involves pyrroloquinoline quinone (PQQ)-dependent enzymes, making it expensive due to the costly cofactor and hence unattractive for industrial applications. In this study, we present a modified PVA film with improved properties as well as a PQQ-independent novel enzymatic cascade for the degradation of modified and unmodified PVA. The cascade consists of four steps catalyzed by three enzymes with in situ cofactor recycling technology making this cascade suitable for industrial applications.

Poly(vinyl alcohol) (PVA) is a water-soluble synthetic vinyl polymer consisting of a carbon-carbon backbone and repeating 1,3-diols. Due to its versatile properties, PVA is used in many industrial applications such as fabric and paper sizing, fiber coating, film for packaging and more.[1] Although PVA was considered a biodegradable polymer, it has become a major pollutant of industrial wastewater.[2] The printing and dyeing industries, in particular, are major PVA emitters. Treatment of PVA wastewater via chemical or physical methods such as adsorption, filtration, ultrasonic degradation or the Fenton reaction are cost intensive, inefficient and can cause secondary pollution. Hence, alternative approaches (e.g., via microorganisms or enzymes) are required for the remediation of wastewater contaminated by PVA.[1] Additionally, in comparison to conventional desizing methods, enzymatic PVA degradation can be carried out under mild conditions.[3] Microbial degradation of PVA was already reported for bacterial strains such as *Pseudomonas putida* VM15 C and VM15 A and *Spingomonas* sp. SA3 and SA2[4] and has been proposed as a two-step process, (i) oxidation of one or two adjacent hydroxyl groups and (ii) subsequent cleavage of the resulting mono- or di-ketone structure. Step one was found to be carried out by a PVA oxidase or a pyrroloquinoline quinone (PQQ)-dependent PVA dehydrogenase (PVA-DH) while an aldolase or a diketone hydrolase catalyze the second step.[5] Kawai and Hu[5b] hypothesized that the polymer is cleaved extracellularly by PVA oxidases which results in fragments small enough to enter the periplasmic space. They base their suggestion on two observations: (i) PVA oxidases were found extracellularly whereas PVA-DHs were discovered membrane bound or periplasmatic[6] and (ii) oxidized PVA can be hydrolyzed by enzymes[7] but can also undergo spontaneous oxidation.[8] In the periplasm, the smaller PVA fragments are further oxidized by the PQQ-dependent PVA-DH. Aldolases or diketone hydrolases subsequently degrade the PVA further until it can be taken up by the organism through the inner membrane.[5b] However, some organisms express only PVA-DH and no PVA oxidases, which suggests that extracellular PVA degradation plays a secondary role.[5b]

Despite the fact that the microbial mechanism for PVA degradation has been known for a long time, research on degradation of PVA with isolated enzymes has been scarce. Further investigation of PVA-DH from *Sphingopyxis* sp. 113 has failed to corroborate initial findings because the recombinantly expressed enzyme formed largely insoluble inclusion bodies.[9] Wei et al., however, further investigated *Stenotrophomonas rhizophila* QL-P4 and were able to efficiently express a novel PVA oxidase.[10] Nonetheless, current literature suggests that PQQ-dependent enzymes are needed for a complete degradation of PVA. Since PQQ is

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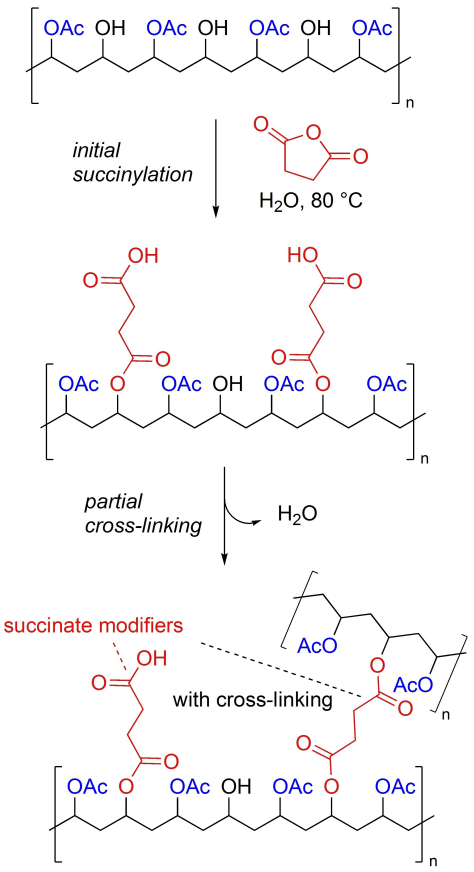
an expensive cofactor, this method appears unattractive for industrial purposes.

In this work, we demonstrate a PQQ-independent enzyme cascade for the degradation of PVA which can act on modified and unmodified PVA. To improve the properties of the PVA polymer, we firstly generated PVA bearing succinylated hydroxyl groups. Specifically, starting from a partially acetylated PVA material was desirable to study new hybrid materials that may find future applications in the packaging industries. Introducing succinate groups on the free hydroxyl groups via simple ester forming reactions was deemed attractive in altering the lipophilicity and mechanical properties in a substrate dependent manner. Succinic anhydride was identified as a desirable modifier that is biorenewable and biodegradable, readily available at low cost and non-toxic.[11] A further advantage is that this protocol avoids the formation of harmful by-products and uses water as the sole solvent. Films modified with different amounts of succinic acid were synthesized after establishing a general protocol for the creation of succinate-modified PVA samples (Scheme 1).



Scheme 1. Synthetic process for the preparation of succinylated PVA. The initial succinylation is carried out in water at 80 °C. Different amounts of succinic anhydride were added to the PVA suspension to yield water-soluble polymers with varying properties. After cooling and evaporation of water, the modified PVA was subsequently partially cross-linked. The Scheme shows an exemplary structure of modified PVA.

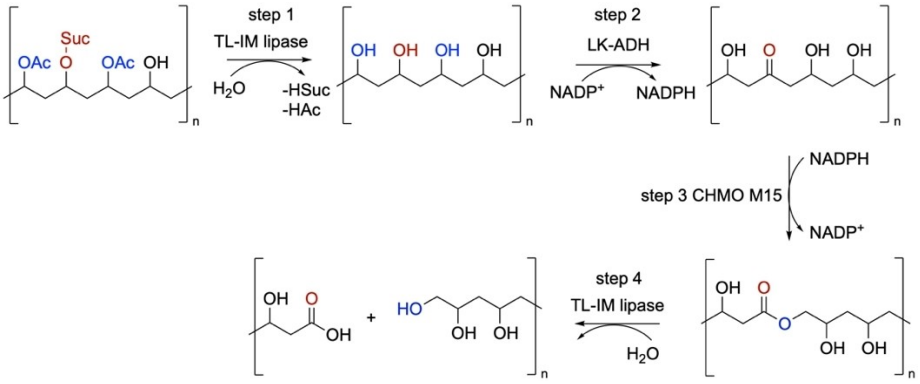
Prior reports on the succinylation of (bio)polymers include cellulose as well as secondary derivatization of the resulting succinate hybrids.[12] This process was exploited using varying amounts of succinic anhydride (2, 4 and 6 g per 30 g PVA) and yielded reproducibly the desired PVA blends. In the following the modified PVA will be referred to as PVA-2 (2 g succinic anhydride), PVA-4 (4 g succinic anhydride) and PVA-6 (6 g succinic anhydride).

Though the focus was on developing a straightforward batch process, initial studies indicated that a continuous flow process is viable which may have advantages when scaling this to the multi-kg scale with regards to reproducibility, automation, safety, and efficiency. Upon casting the aqueous hybrid materials into plastic boxes and slow evaporation of water in a fume hood, thin polymer films were obtained that could be easily peeled and used for further analysis. Infrared spectroscopy was used as a technique of choice indicating a distinct change in the carbonyl stretching region (i.e., 1600–1800 cm^{-1}) which is in agreement with the introduction of succinate moieties; i.e., 1714 cm^{-1} for unmodified PVA vs. 1703 cm^{-1} for the succinate hybrid (Supporting Information, Figure S1).

Further and more comprehensive testing was performed externally and indicated that key parameters can be altered effectively and in dependence of the amount of succinic anhydride used (Supporting Information, Table S3). As such, all succinate hybrids were characterized by excellent mechanical properties and retained the water solubility/dispersibility of the original PVA. These parameters are critical for future use as packaging materials, and the ability to dissolve in aqueous environments is expected to expedite biodegradation which is also supported by a slow onset of disintegration in soil (Supporting Information, Table S3). However, the oxygen and water vapor transmission of the modified PVAs were significantly higher in comparison to the values for unmodified PVA (Supporting Information, Table S3).

Next, we developed an enzymatic cascade for the degradation of the modified PVA using three biocatalysts. This cascade can be performed as a one pot reaction including an in situ cofactor recycling. All enzymes used are well characterized and either easily recombinantly expressible in *Escherichia coli* or commercially available.[13] The enzyme cascade consists of four steps (Scheme 2) for modified PVA but can also be applied for unmodified PVA, starting at step 2. In step 1, ester bonds in the derivatized side chains are hydrolyzed by the commercially available lipase TL-IM resulting in unmodified PVA. The lipase is described to be 1,3-specific and originates from *Thermomyces lanuginosus*. Additionally, the immobilized enzyme accepts substrates with bulky side chains, making it an ideal candidate for the PVA degrading enzyme cascade.

After cleaving the ester bonds, the hydroxyl groups are oxidized to ketones by an alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) utilizing the cofactor $NADP^+$ with formation of NADPH (Scheme 2, step 2).[13a] These ketones are then further oxidized to esters by the cyclohexanone monooxygenase (CHMO) variant M15 originally from the organism *Acinetobacter calcoaceticus*.



Scheme 2. Enzyme cascade for the degradation of modified and unmodified PVA. In step 1, the commercial lipase TL-IM removes the esters (HAc and HSuc) from the PVA via hydrolysis which results in unmodified PVA with 1,3-diol functional groups. These diols are then oxidized into ketones by LK-ADH, using $NADP^+$ as the cofactor and converting it into NADPH. NADPH is the cofactor for the subsequent enzymatic Baeyer–Villiger oxidation of ketones into ester-bonds which is performed by CHMO M15. In the last step, the ester bond is cleaved, which results in smaller PVA fragments. For PVA without modification, the same cascade can be applied, starting then from step 2. For simplicity, only one distinct example cascade pathway is shown, of course, steps 2–4 can also occur at further functional groups.

CHMO M15 is utilizing the cofactor NADPH with formation of $NADP^+$.[13b] This, in principle, leads to an advantageous in situ cofactor recycling. Finally, the newly formed ester bond can be cleaved by lipase TL-IM through hydrolysis which results in smaller fragments of the polymer. Hence, a total of three enzymes in a one pot cascade can cleave PVA to release oligomers that are more accessible to further degradation by e.g., microorganisms or may serve as building blocks for upcycling of the resulting material.

Degradation of PVA can be measured by staining the polymer using iodide. According to the Finley method, PVA forms a green complex with iodide in the presence of boric acid with an absorption maximum at 500 nm.[14] Changes in the staining of PVA due to degradation of the polymer can thus be detected by absorbance measurements. PVA was incubated with crude cell lysates from *E. coli* containing LK-ADH and CHMO M15 as well as the immobilized TL-IM in a one pot reaction. As negative control, cell lysate without overexpressed enzyme was used to exclude an influence of the lysate on the polymer.

The enzyme cascade caused a decrease in absorbance of the derivatized PVA film solution at 500 nm compared to the negative control when investigated with the Finley method. The degradation performance of PVA-4 film was evaluated at a wide range of pH, temperatures, reaction times, enzyme ratios and amounts of cofactor (Figure 1). To

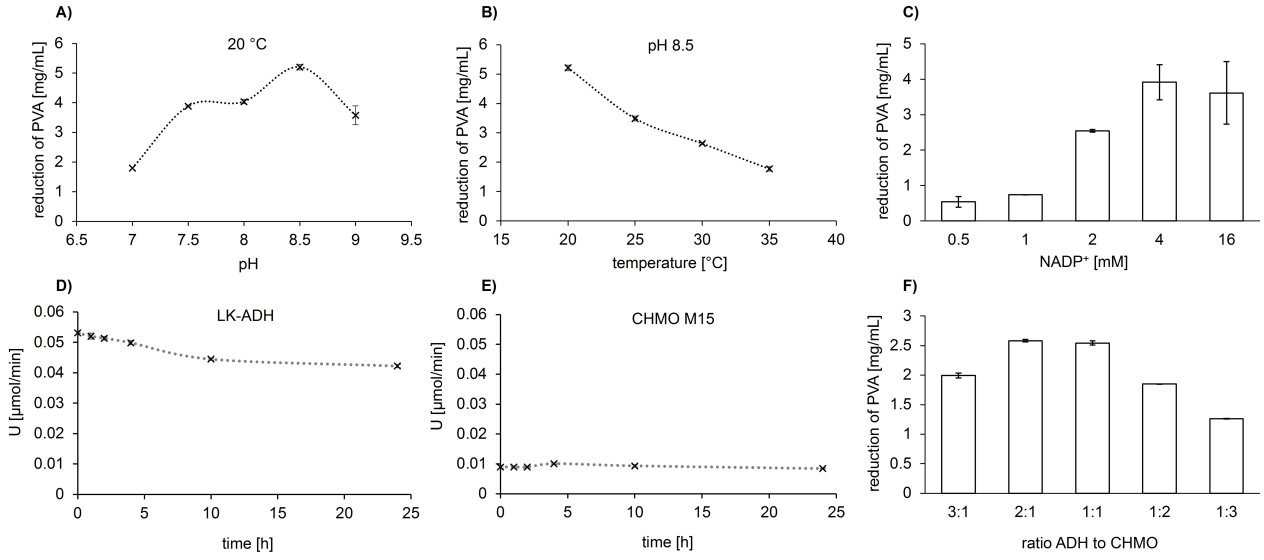


Figure 1. Investigation of the effect of various conditions on the enzymatic degradation of film PVA-4. The reaction conditions were optimized regarding pH (A), temperature (B), the amount of cofactor (C), the reaction time (D & E), and the ratio of ADH to CHMO (F). The best conditions were found to be pH 8.5, 20 °C, 24 h with 4 mM cofactor $NADP^+$. D) The activity of LK-ADH was measured by following the increase of NADPH when converting (*R*)-1-phenyl ethanol. E) The activity of CHMO M15 was measured by following the decrease of NADPH when converting cyclohexanone. One Unit (U) was defined as the amount of substrate converted per minute by one µL of enzyme lysate.

investigate the temperature range of the enzymes used for the cascade, each enzyme was incubated for one hour at varying temperatures (4–50 °C). Subsequently, the residual activity of each enzyme was measured and plotted (Supporting Information, Figure S2). It was found that LK-ADH and CHMO M15 are less stable in comparison to the immobilized TL-IM. These results are in agreement with the enzyme description for the TL-IM provided by the manufacturer Novozymes. According to the manufacturer, TL-IM has a temperature optimum of 50–75 °C. Thus, steps 2 and 3 of the PVA degradation (Scheme 2) are considered the crucial steps in the enzyme cascade.

Next, the pH of the reaction was varied in a range from 7 to 9 and, based on the results shown in Figure S2, different temperatures for the cascade reaction were studied in a range from 20 to 35 °C. It was found that the enzymatic degradation of PVA-4 ideally takes place at pH 8.5 and 20 °C under the tested conditions (Figures 1A and B). The time dependent stability of LK-ADH and CHMO M15 was investigated at 20 °C and pH 8.5 over 24 h by following the increase or decrease of NADPH using the substrates (*R*)-1-phenyl ethanol (LK-ADH) or cyclohexanone (CHMO M15) (Figures 1D and E). While CHMO M15 retained 80 % of its activity for the duration of the experiment, the activity of LK-ADH decreased by 20 % in 24 h at 20 °C. Hence, the degradation of PVA by the enzyme cascade can be performed at 20 °C over 24 h without a major loss of enzymatic activity. Furthermore, the optimal amount of cofactor for the reaction was investigated. Since the reaction contains an in situ cofactor recycling system and in step 2 $NADP^+$ is firstly required by LK-ADH for the formation of ketones, only $NADP^+$ was added to the reaction. It was found that the efficiency of the reaction increases with increasing amounts of $NADP^+$ up to 4 mM. However, the reaction efficiency did not improve when more than 4 mM $NADP^+$ were added to the enzyme cascade (Figure 1C).

Lastly, for a sufficient cofactor recycling system, the optimal ratio of LK-ADH to CHMO M15 was investigated for the degradation of PVA film PVA-4. From the Finley method we found that enzyme ratios of 1:1 or 2:1 were most favorable for the enzymatic reaction (Figure 1F). It should be mentioned at this point that the enzyme ratio and the amount of cofactor are most likely interdependent. The investigation of the enzyme ratio was performed with 4 mM $NADP^+$ whereas the investigation of the optimal amount of cofactor was carried out with a 1:1 LK-ADH to CHMO M15 ratio. In the stability testing of the enzymes, shown in Figure 1, LK-ADH performed with a higher activity (0.053 U per µL) compared to CHMO M15 (0.009 U per µL) which could lead to the conclusion that LK-ADH will convert $NADP^+$ faster into NADPH than CHMO M15 converts NADPH into $NADP^+$. However, the activity assay was carried out with the substrates (*R*)-1-phenylethanol and cyclohexanone and the activity for those substrates cannot be directly transferred to PVA. Next, the enzyme ratios for PVA degradation were investigated with 2 mM $NADP^+$ (data not shown) and 4 mM $NADP^+$ which led to similar conclusions regarding the optimal enzyme ratio.

Thus, the improved degradation reaction of PVA-4 with the proposed enzyme cascade contained 4 mM $NADP^+$, LK-ADH and CHMO M15 in a ratio of either 1:1 or 2:1 carried out at pH 8.5 and 20 °C over 24 h.

Using gel permeation chromatography (GPC) the molecular mass of PVA before and after enzymatic treatment was also explored. Here, in addition to PVA-4, unmodified PVA as well as PVA-2 and PVA-6 were investigated as well for degradation with the enzyme cascade under the above-mentioned conditions. The polydispersity index (PDI), the number average molecular weight (M_n) and the weight average molecular weight (M_w) were calculated from the measurements (Table 1). PDI increased in most conditions tested when the PVA film was treated with enzymes, indicating a broadening of the molecular weight distribution in comparison to the untreated films which in turn is an indicator for degradation of the polymer. Additionally, a decrease in M_n and M_w was observed in samples containing

Table 1: Average M_n and M_w of PVA-films determined for unmodified PVA, PVA-2, PVA-4 and PVA-6 degraded by the enzyme cascade. The average and the standard deviations were calculated from three replicates.

LK-ADH to CHMO ratio	Film	Treatment	M_n [g mol^{-1}]	M_w [g mol^{-1}]	PDI[a]
1:1	Unmodified PVA	Enzyme cascade	37850 ± 144	161767 ± 2970	4.27
		Negative control	40667 ± 230	168167 ± 2702	4.14
	PVA-2	Enzyme cascade	33407 ± 190	98373 ± 410	2.94
		Negative control	35607 ± 163	101433 ± 814	2.85
	PVA-4	Enzyme cascade	35719 ± 663	105867 ± 2581	2.96
		Negative control	39527 ± 215	111367 ± 1380	2.82
	PVA-6	Enzyme cascade	34823 ± 704	106600 ± 800	3.06
		Negative control	38533 ± 135	11046 ± 3044	2.87
2:1	Unmodified PVA	Enzyme cascade	36147 ± 1809	145267 ± 424	4.02
		Negative control	39823 ± 351	161200 ± 2425	4.05
	PVA-2	Enzyme cascade	34030 ± 177	97460 ± 438	2.86
		Negative control	35683 ± 121	101267 ± 473	2.84
	PVA-4	Enzyme cascade	35177 ± 232	103120 ± 3201	2.93
		Negative control	39060 ± 170	110500 ± 2252	2.83
	PVA-6	Enzyme cascade	34197 ± 770	104200 ± 2128	3.05
		Negative control	37541 ± 1293	109133 ± 2011	2.91

[a] Polydispersity index.

the enzyme cascade in comparison to the negative control. When PVA samples were treated with the enzyme cascade, both, M_n and M_w decreased in comparison to untreated PVA. As already observed with the Finley method, the GPC measurements confirmed that the enzyme ratio 1:1 or 2:1 result in a similar reduction of M_n and M_w and hence, that changing the ratio of enzyme from 1:1 to 2:1 does not influence the reaction significantly. The largest decrease in M_n was observed in degradation reactions with film PVA-4. Independently of the LK-ADH to CHMO ratio, M_n decreased by 10% when PVA-4 was treated with enzyme. The largest decrease in $M_{w,}$ however, was measured for unmodified PVA. When using a 2:1 LK-ADH to CHMO ratio, M_w decreased by 10% for unmodified PVA whereas for PVA-4 it only decreased by 7%. In general, the proportional decrease of M_w was lower in comparison to M_n in most cases. The exception to this is the PVA film without modification. Using an enzyme ratio of 2:1, M_w was reduced by 10% and M_n by 9% when the film was incubated with the enzyme cascade. In comparison to that, an enzyme ratio of 1:1 reduced M_w by only 4% and M_n by 7% indicating that the enzyme ratio of LK-ADH to CHMO M15 has a higher impact on PVA without modification in comparison to modified PVA. This could be due to the additional step in the reaction of modified PVA for hydrolyzing the ester bonds in the derivatized side chains of the polymer which delays the subsequent reactions of ADH and CHMO in comparison to unmodified PVA. Enhancement of enzyme stability of ADH and CHMO as well as prolonged reaction time could hence potentially lead to similar yields for modified PVA as were gained for unmodified PVA. Furthermore, from a larger decrease of M_w in comparison to M_n it can be concluded that larger molecules were more readily degraded when an enzyme ratio of 2:1 was used for unmodified PVA. M_w is taking the weight of each polymer chain into account and hence the value is less influenced than M_n when smaller molecules are preferentially degraded. This in turn leads to the conclusion that larger amounts of LK-ADH are needed for the degradation of unmodified, larger weight PVA molecules. Even though LK-ADH was proven to tolerate a wide variety of substrates and to also accept bulky side chains,[15] the polymer is most likely challenging for the enzyme and an increase of the enzyme amount could hence help with degrading larger molecules. Enzyme engineering of LK-ADH towards acceptance of larger side changes could further improve the degradation rate of PVA.

In summary, a modification of PVA was achieved with succinic anhydride. This was realized via a simple chemical process using water as solvent at elevated temperature rendering succinylated PVA films through wet casting techniques. Most importantly, the here described novel enzyme cascade consisting of four steps carried out by three enzymes can break down the modified PVA with improved properties as well as PVA without modification. The in situ recycling of the cofactor NADP(H) makes this enzyme cascade self-sufficient, more versatile, and applicable in comparison to published (microbial) systems for PVA degradation. Notably, the efficiency of the in situ cofactor recycling between LK-ADH and CHMO was already reported for the conversion of cyclohexanol into ε-caprolactone.[16]

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

Keywords: Degradation · Enzyme Cascade · Enzyme Catalysis · Plastic · Poly(Vinyl Alcohol)

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