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Research Article

Repurposing Commercial Hydrolytic and Oxidative Enzymes toward Synergistic PLA Depolymerization

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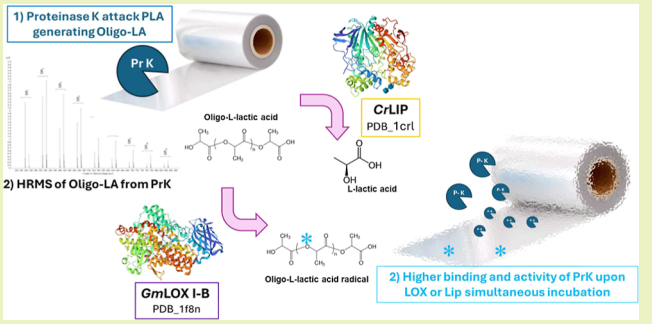
ABSTRACT: The enzymatic depolymerization of polylactic acid (PLA) under mild conditions remains a major challenge in sustainable plastic recycling. Here, we report a synergistic system combining *Tritirachium album* proteinase K (PrK) with two commercial biocatalysts, *Candida rugosa* lipase (Lip) and *Glycine max* lipoxygenase (LOX I-B), that enhances PLA degradation far beyond individual enzyme performance. PrK alone hydrolyzed amorphous solvent-cast PLA ($_{V}$PLA) and postconsumer PLA ($_{PC}$PLA) with moderate efficiency, but the addition of Lip led to a 5.7-fold increase in lactic acid (LA) release on $_{V}$PLA and a 2.7-fold increase on $_{PC}$PLA, achieving ∼60% conversion within 72 h. Similarly, LOX I-B, while catalytically inactive on its own, boosted PrK-mediated $_{V}$PLA hydrolysis up to 2.5-fold, dependent on molecular oxygen, and yielded pyruvate as a trace oxidative product. Mechanistic investigations revealed that both Lip and LOX I-B promote PrK binding to PLA surfaces, enhancing productive interactions with the polymer. Additionally, Lip hydrolyzed soluble oligo-lactide (O-LA) intermediates generated by PrK, contributing to monomer release. High-resolution mass spectrometry and FTIR spectroscopy confirmed substrate-dependent synergy, surface modifications, and the progressive removal of O-LA species. The study demonstrates that enzymatic cocatalysis using trace amounts of auxiliary hydrolases or redox enzymes can significantly accelerate PLA depolymerization. These findings establish a mechanistic framework for multienzyme systems in polyester biodegradation and offer a promising biocatalytic strategy for scalable PLA recycling.



KEYWORDS: *PLA-oxidation, proteinase-K, lipase, lipoxygenase, oligo-PLA*

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1. INTRODUCTION

Polylactic acid (PLA) is a biobased aliphatic polyester produced from renewable resources. Its building block is lactic acid (LA), which is mainly produced through fermentation processes rather than chemical synthesis, as fermentation enables the production of optically pure D-LA or L-LA, instead of a racemic mixture.[1] Additionally, LA fermentation has the potential to use nonfood sources as feedstock, such as lignocellulosic biomasses (LCB), reducing competition with food supplies,[2] such as sugars and starch. LA produced via fermentation can be polymerized either through the polycondensation of the LA monomer or by ring-opening polymerization (ROP) of its cyclic dimer, lactide, to form poly-L-LA (PLLA), poly-D-LA (PDLA), or poly-D,L-LA (PDLLA). While polycondensation is less expensive to operate, it generates water as a byproduct, which limits the final molecular weight of the polymer. Consequently, ROP is the preferred industrial method as it allows the production of solvent-free polymers with higher molecular weights. However, the additional steps required for lactide synthesis and purification result in higher production costs for PLA compared to petroleum-based plastics.[3] ROP also enables the controlled incorporation of D- and L-LA monomers, facilitating the tuning of the polymer properties. For instance, the inclusion of a small percentage of D-LA decreases the melting temperature (T_m) and the degree of crystallinity of the polymer,[4] improving its performance during melt-processing conversion.[5]

PLA is the most widely produced bioplastic, with a global production volume of approximately 1 million tons in 2024, which is projected to increase 2.6-fold by 2029.[6] PLA is utilized across various sectors, including packaging, textiles, and biomedical applications, owing to its biocompatibility and the nontoxic nature of its degradation products. However, its applicability is limited by brittleness, low thermal stability,

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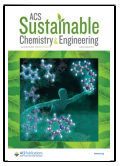

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hydrophobicity, and its low rate of biodegradation in vivo.[7] In fact, PLA requires high temperatures, close to its T_g (i.e., 55−60 °C), to be efficiently depolymerized. In marine and terrestrial ecosystems, as well as in urban composting facilities where temperatures remain lower than PLA's T_g, its degradation can take decades to complete. Only under controlled conditions of industrial composting facilities, where temperatures exceed 60 °C, PLA can be completely broken down within 180 days.[8] Given PLA's slow degradation rate in the environment and its high production cost, implementing recycling and upcycling strategies is recommended as a more sustainable approach for managing end-of-life (EoL) PLA products.[9] To do so, over the past two decades, significant research efforts have been dedicated to the discovery and characterization of EoL-PLA degrading enzymes. For instance, Guicherd et al. (2024)[10] demonstrated that the incorporation of an engineered enzyme into PLA enables its rapid biodegradation under home-composting conditions.

PLA depolymerases belong to the serine hydrolase class and include proteases (EC 3.4.-), which specifically target PLLA,[11] as well as carboxylesterases, lipases, and cutinases (EC 3.1.1.-), which preferentially hydrolyze PDLLA. PLA-active proteases are promiscuous enzymes capable of degrading PLLA due to its structural similarity to silk fibroin, which consists of L-alanine, i.e., a structural analogue to L-LA.[12] Williams (1981)[13] was the first to report enzymatic degradation of PLA, identifying Proteinase K from *Tritirachium album* as an effective enzyme, which continues to serve as a gold standard for PLA degradation research. Among commercially available enzymes, Oda et al. (2001)[14] screened 56 proteases and identified alkaline proteases as the most effective in hydrolyzing PLA, linking their activity to keratin-degrading properties. Additionally, the search for PLA depolymerases has extended beyond commercial enzymes, focusing on the isolation of microbial enzymes from culture supernatants[15] and metagenomic mining approaches.[16,17] Fewer commercial lipases[18,19] have been characterized for PLA hydrolysis, and limited studies have investigated the synergistic interactions between lipases and proteases in the context of PLA degradation.[20]

In this work, three commercial enzymes, Proteinase K (PrK) from *T. album*, *Candida rugosa* lipase (CrLip), and *Glycine max* lipoxygenase (LOX I-B), were investigated for their synergistic action for the degradation of solvent-cast, as well as EoL-PLA films. While Lip and LOX I-B alone did not promote PLA hydrolysis, they significantly enhanced PrK-mediated hydrolysis by 5.7-fold and 2.5-fold, respectively. Lip, and to a lesser extent LOX I-B, increased PrK's productive binding to the polymer, thus accelerating catalysis. These findings underline the potential of synergistic enzymes for PLA degradation and provide the first evidence of lipoxygenase activity in this process. This approach provides a solid foundation for the development of more efficient enzymatic cocktails for recycling and upcycling of PLA, offering sustainable alternatives for the valorization of plastic waste.

2. MATERIALS AND METHODS

2.1. Materials and Enzymes. All reagents used in this study were purchased from Carl Roth (Karlsruhe, Germany) and were of analytical grade, unless otherwise specified. Virgin PLA ($_V$PLA) films were prepared as described in Section 2.2 using Luminy LX175 PLA pellets (Corbion). Postconsumer PLA ($_{PC}$PLA) was obtained from surgical mask packaging wrap. The enzymes used in this study included lipase from *C. rugosa* Type VII (CAS: 9001-62-1) and type I-B lipoxygenase from *G. max* (CAS: 9029-60-1), both purchased from Sigma-Aldrich (St. Louis, MO, USA). Proteinase K (CAS: 39450-01-6) was obtained from Carl Roth.

2.2. PLA Film Production. PLA films were prepared using the solvent-casting method, following the protocol described by Decorosi et al. (2019)[21] with minor modifications. Briefly, 2 g of PLA pellets was dissolved in 50 mL of dichloromethane under continuous stirring until complete dissolution (approximately 30 min). Subsequently, 25 mL of the resulting solution was transferred into a polypropylene container (10.5 × 16 cm), and the solvent was allowed to evaporate at room temperature overnight. While we refer to the material used in this work as PLA, it should be noted that the exact composition is a PLLA for 98−97% containing a few percents of D-LA 2−3% as measured (see next paragraph).

2.3. Enzymatic Degradation of PLA by Commercial Enzymes. Three commercial enzymes, the Proteinase K (PrK) from *T. album*, a lipase from *C. rugosa* (CrLip), and a lipoxygenase from *G. max* (LOX I-B), were evaluated for their ability to degrade $_V$PLA and $_{PC}$PLA films, which were cut into 0.5 cm × 0.5 cm square pieces. All reactions were conducted in 100 mM sodium phosphate buffer (pH 7.2 ± 0.2) containing 1% (w/v) PLA as the substrate. The effect of calcium (Ca^{2+}), a known activating cation, on PLA degradation was assessed by supplementing the reaction mixture with $CaCl_2$ at a final concentration of 0.1 mM.

Lyophilized enzymes were dissolved in the reaction buffer to prepare 100× concentrated stock solutions. Reactions were initiated by adding the enzymes to achieve final concentrations of 0.43 mg/mL for PrK, 30 μg/mL for Lip, and either 36 μg/mL or 0.15 μg/mL for LOX I-B. The concentration of PrK was selected based on previously reported studies,[22,23] while Lip and LOX were applied at lower concentrations to evaluate their potential synergistic effects on PrK-mediated PLA depolymerization. The reaction mixtures were incubated at 50 °C with constant shaking at 750 rpm for up to 72 h. Reactions were stopped by centrifugation at 10,000*g* for 5 min. The supernatants were subsequently filtered using 0.22 μm polytetrafluoroethylene (PTFE) membranes and analyzed via HPLC. To remove oxygen (O_2), the reaction mixtures were boiled at 90 °C for 10 min and cooled before enzyme addition, and the headspace of the reaction tubes was flushed with carbon dioxide (CO_2) during enzyme stock addition. The remaining PLA pieces after 24 h incubation with each respective enzyme or the negative control without enzymes were removed, washed thoroughly in fresh buffer, and freeze-dried for FTIR analysis.

The experiment for the detection of O-LA was set as all the others except that after 24 h the PLA substrate was removed, and a second enzyme or neither was added. The conditions, enzyme dosages, temperature, and stirring were kept the same as described above.

D-LA was quantified using the D-lactic acid assay kit (K-DATE, Megazyme), following the manufacturer's instructions. Total LA released during the reactions was quantified using an UltiMate 3000 UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA), equipped with a photodiode array detector set at 210 nm. Chromatographic separation was performed at 65 °C using a Rezex ROA-Organic Acid H^+ column (Phenomenex, Torrance, CA, USA) under isocratic conditions, using 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min. A calibration curve was prepared with LA standard solutions ranging from 8 to 0.125 g/L.

Results were expressed as the percentage of film degradation calculated by dividing the amount of LA released during the reaction by the maximum theoretical yield of LA (LA_{MAX}), as shown in eq 1.

$$\%\ \text{PLA degradation} = \frac{\text{LA}}{\text{LA}_{\text{MAX}}} \times 100 \quad (1)$$

LA_{MAX} was calculated for each reaction as the total amount of LA released assuming complete hydrolysis of the film. The value was normalized to account for the addition of a water molecule during the hydrolysis of the ester bond, which leads to an increase in the

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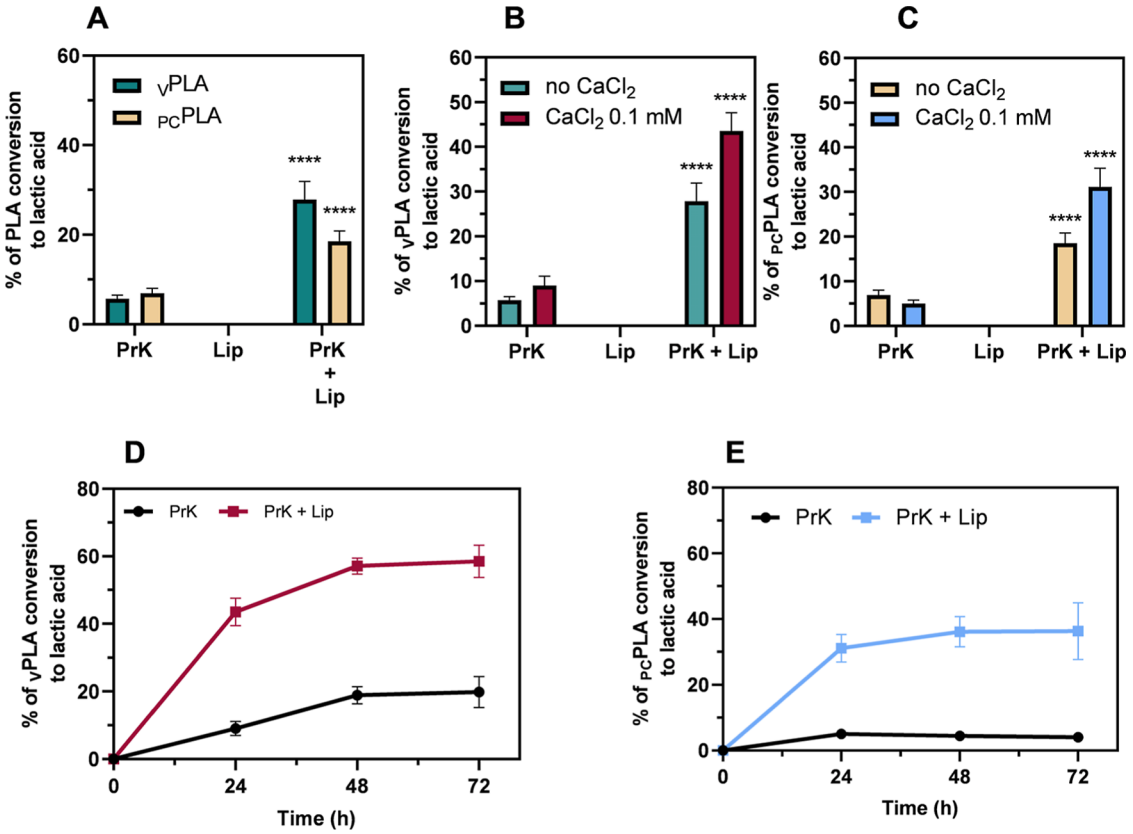

Figure 1. PLA film hydrolysis mediated by PrK and Lip. (A) Synergistic effect of Lip in combination with PrK on $_{V}$PLA and $_{PC}$PLA. (B) The effect of calcium on the PrK- and Lip-mediated degradation of $_{V}$PLA. (C) The effect of calcium on the PrK- and Lip-mediated degradation of $_{PC}$PLA. (D) Synergistic effect of Lip in combination with PrK on $_{V}$PLA over 72 h of incubation with 0.1 mM $CaCl_2$. (E) Synergistic effect of Lip in combination with PrK on $_{PC}$PLA over 72 h of incubation with 0.1 mM $CaCl_2$. Enzyme concentrations used: PrK 0.43 mg/mL and Lip 30 μg/mL. Every data point was collected at least in triplicate and synergism was tested using randomized two-way ANOVA.

molecular weight of the monomer released compared to the monomeric unit within the polymer, as described in eq 2.

$$LA_{MAX}\ (g/L) = \frac{m_{PLA}}{V} \times 1.25 \quad (2)$$

2.4. Adsorption Assay. The adsorption of enzymes to the PLA surface was monitored during the reaction by measuring the concentration of unbound protein in the supernatant. To achieve this, multiple reactions were prepared by following the same experimental conditions described in the previous section. Aliquots of the reaction mixture were collected at the start and at the following time points (hourly in the range of 1−6 h and at 20 h) to assess the free enzyme concentration. The protein concentration in the supernatant was quantified using the Bradford reagent (Bio-Rad, California, USA), according to the manufacturer's instructions.

2.5. Pyruvic Acid and Oligo-Lactide Detection and Quantification. High-resolution mass spectrometry (HRMS) analysis was performed for the targeted qualitative detection of pyruvic acid (PA: $C_3H_4O_3$) and oligo-lactides (O-LA) in the supernatant of the PLA incubations with enzymes. For this purpose, HRMS data were acquired using a 1260 series rapid resolution liquid chromatography (RRLC) and an Agilent Q-TOF 6546 MS detector, both from Agilent Technologies. For the aqueous solvent, 0.2% of formic acid with Milli-Q water was used and (80:20, V:V) of (methanol/aqueous solvent) as the organic solvent. The injected volume was adjusted to 20 μL. The run lasts 1.5 min with the following composition (aqueous solution 37.5%, organic solvent 62.5%). An electrospray ionization (ESI) source in positive mode was used with the following settings: 320 °C source temperature, 8 L/min drying gas flow, 35 psi nebulizer pressure, 3.5 kV capillary voltage, and 175 V fragmentor voltage. The mass range was defined from 100 to 3200 m/z with an acquisition rate of 1 spectra/s using the centroid mode for the data storage. All data were acquired in MS scan mode and high-resolution range (10 GHz) mode with Agilent Mass Hunter LC/MS Acquisition 11.00 and treated with Agilent Mass Hunter Qualitative analysis B.12.00.

HPAEC-PAD was used for the quantification of PA. The separation was achieved with a CarboPac PA1 column (4 mm diameter × 250 mm length) while the eluent program was run accordingly.[24] The detection was acquired with a pulsed amperometric sensor (PAD). A calibration curve was prepared with PA standard solutions ranging from 250 to 1 mg/L.

2.6. FTIR of PLA. Fourier transform infrared spectroscopy was performed using a Tensor27 infrared spectrophotometer (Bruker, Billarica) equipped with an MCT detector cooled with liquid nitrogen at a resolution of 4 cm^{-1}. The samples were previously freeze-dried. PLA films were washed with Na-phosphate buffer at pH 9.0, 1 M, for few seconds and re-equilibrated in Milli-Q water before freeze-drying. The sample was pressed on a diamond crystal (golden gate from Specac Orpington) to acquire the spectra by attenuated total reflection (ATR). Each spectra represent a normalized and average of 3 individual samples, with 64 scans for each sample.

2.7. ROS Detection during PLA Incubation with Enzymes. The Amplex Red method was adopted for the detection of ROS during the incubation of PLA with enzymes. Production of H_2O_2 was quantified using the MyQubit Amplex Red Peroxide Assay (Thermo Fisher Scientific) following the manufacturer's protocol. The working solution (WS) contained 100 μmol/L Amplex Red reagent, 0.2 U/mL of horseradish peroxidase (HRP), and 1× phosphate buffer saline (PBS) at pH 7.4. 100 μL of the sample was incubated with 100 μL of WS and 40 μL of PBS during 5 min in the dark. Resorufin fluorescence was measured using Qbit 3 Fluorometer (Thermo Fisher

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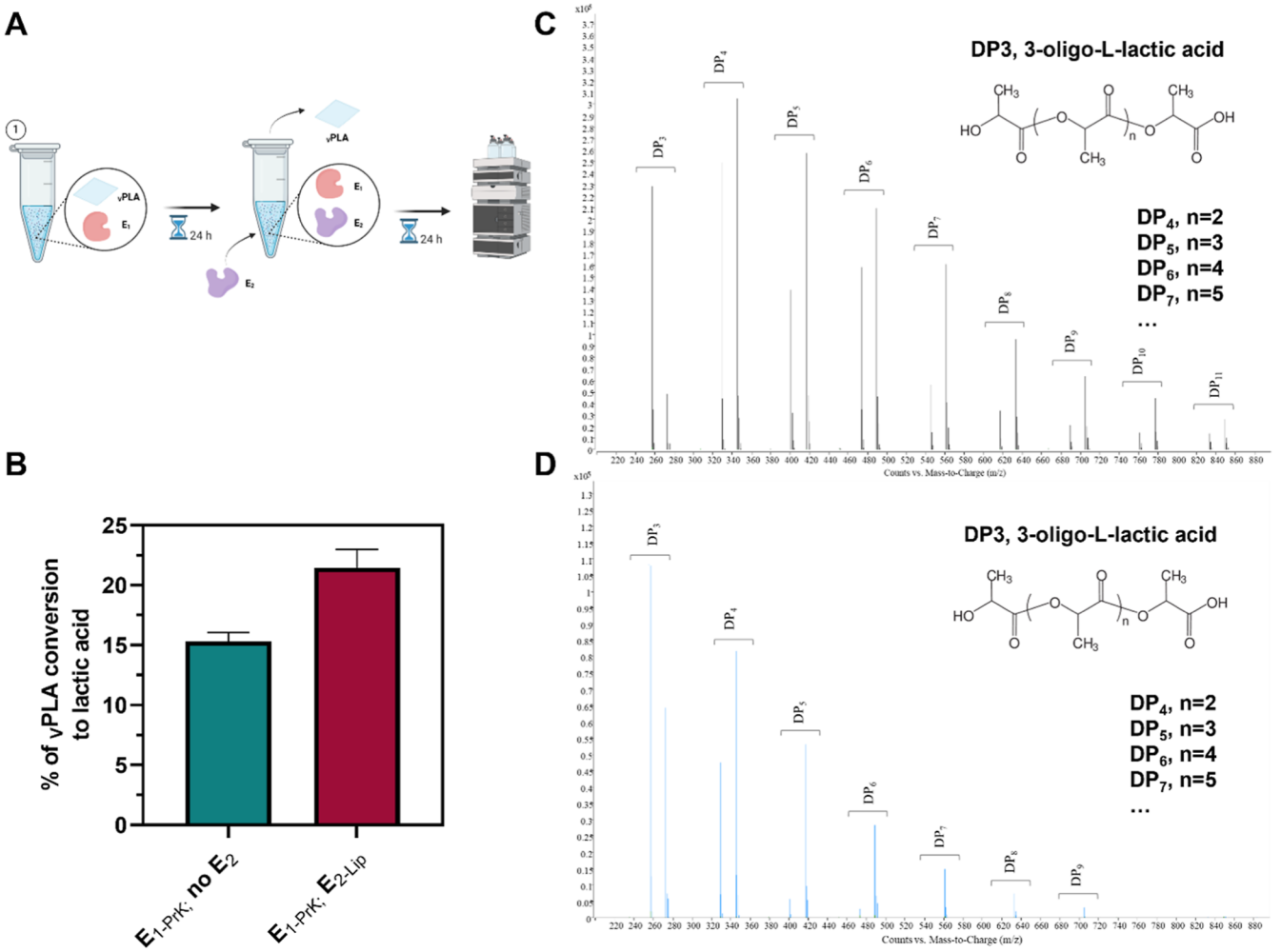

Figure 2. O-LA release during $_{V}$PLA degradation with PrK and Lip. (A) Schematic drawing relative to the experimental set up: $_{V}$PLA was first incubated for 24 h in the presence of $CaCl_2$ with enzyme-1 PrK (E1-PrK), successively the remaining of the film was removed and in the supernatant enzyme-2 was added (E2-Lip) or not (no E2); the reaction was incubated at 50 °C for another 24 h. All the other conditions were kept equal as already described for previous hydrolysis with PrK. (B) Quantification of LA by HPLC in the 48 h sample relative to the setting without addition of a E2 (green bar) or with addition of Lip as E2 (red bar). The data are reported as % of maximum theoretical degradation of $_{V}$PLA. (C) HRMS analysis of oligo-lactides (O-LA) from DP3 to DP11 relative to the supernatant relative to the 48 h incubation of $_{V}$PLA incubate with PrK (E1-PrK; no E2), the exact masses are reported in Supporting Information Figure S1. (D) HRMS analysis of O-LA relative to incubation of $_{V}$PLA with E1-PrK; E2-Lip enzymatic hydrolysis setting, the exact masses are reported in Supporting Information Figure S3.

Scientific) and automatically converted into H_2O_2 using a calibration curve ranging from 0 to 25 μM.

2.8. Statistical Analysis. All data were collected at least in triplicate and are presented as the mean ± standard deviation. A two-tailed, completely randomized two-way ANOVA was performed to assess the statistical significance of synergistic effects within the data sets. The significance level was set at 0.05 (*), with ** indicating p value (p) ≤ 0.01; *** for $p \leq 0.001$, and **** for $p \leq 0.0001$.

3. RESULTS

3.1. Proteinase K and *C. rugosa* Lipase Show Synergism in PLA Depolymerization. The PLA-depolymerizing activity of Proteinase K (PrK) from *T. album* and *C. rugosa* lipase (CrLip) was tested on solvent-cast ($_{V}$PLA) and postconsumer PLA ($_{PC}$PLA) films. PLA degradation was enhanced at temperatures close to its T_g due to the increased chain mobility, which facilitated enzymatic hydrolysis of the ester bonds. Accordingly, the incubation temperature was set at 50 °C, which was close to the T_g of PLA (55−60 °C) and within the previously reported optimal temperature ranges of PrK[25] and Lip.[26] Both enzymes exhibited activity in neutral and alkaline pH ranges. Therefore, assays were performed at a pH of 7.2 to minimize the contribution of base-catalyzed hydrolytic side reactions that occur at alkaline pH values.[27]

As shown in Figure 1A, PrK exhibited comparable yet modest activity on the two PLA substrates tested, yielding a conversion to LA of 5.7% for $_{V}$PLA and 6.9% for $_{PC}$PLA following a 24 h incubation timespan. In contrast, LA release was not observed when Lip was incubated with either of the PLA substrates, which is consistent with the findings reported by Hegyesi et al., (2019).[28] HRMS analysis revealed also a certain level of oligo-lactide (O-LA) being released during the incubation of PrK when incubated with $_{V}$PLA (Figure 2). Nonetheless, despite its inability to release LA when acting as a standalone enzyme, Lip exerted a significant synergistic effect when combined with PrK, enhancing the release of LA by 5.7-fold for $_{V}$PLA and 2.7-fold for $_{PC}$PLA, perhaps targeting the O-LA released by PrK.

PrK activity is influenced by calcium, which, although not directly involved in catalysis, affects the enzyme's three-

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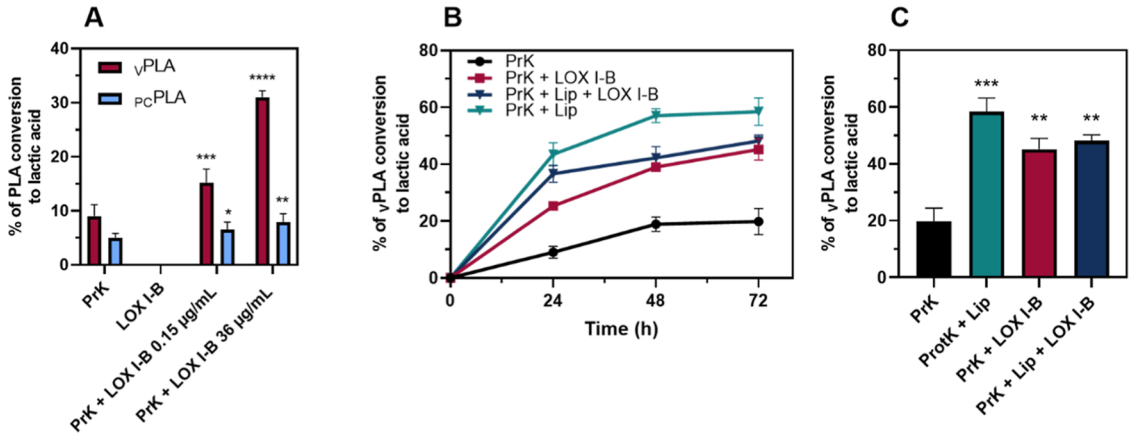

Figure 3. PLA film hydrolysis mediated by PrK and LOX I-B. (A) Synergistic effect of LOX I-B in combination with PrK on $_{V}$PLA and $_{PC}$PLA. (B) Time course reactions of combinations of PrK with LOX and Lip, showing the $_{V}$PLA conversion yield into LA. (C) Specific conversion yield obtained at 72 h for all the tested combinations of the enzymes. All reactions were carried out in 100 mM sodium phosphate buffer at pH 7.2, supplemented with 0.1 mM $CaCl_2$ and 1% (w/v) $_{V}$PLA film substrate. Enzyme concentrations were as follows: PrK at 0.43 mg/mL, Lip 30 μg/mL, and LOX I-B 36 μg/mL (if not otherwise stated). All data were collected in triplicate and synergism was tested using randomized two-way ANOVA.

dimensional structure. Its absence triggers a concerted conformational change, which results in reduced catalytic activity.[29] To address this, the effect of adding Ca^{2+} ions in the form of 0.1 mM $CaCl_2$ was tested (Figure 1B). Interestingly, the addition of $CaCl_2$ resulted in a 36% increase in activity of PrK toward $_{V}$PLA both in the presence and absence of Lip (Figure 1B). Furthermore, Lip continued to exhibit a significant synergistic effect when combined with PrK in the presence of $CaCl_2$, achieving a total conversion of 43.5% of $_{V}$PLA to LA within a 24 h incubation period. The extension of the reaction period led to a plateau in the combined activity of PrK and Lip, achieving approximately 60% substrate degradation in 48 h (Figure 1D) and representing a 3-fold increase compared to the activity observed with PrK alone.

In the case of $_{PC}$PLA, the addition of $CaCl_2$ resulted in a 70% increase in PrK's degradation efficiency in combination with Lip, yielding a final conversion of 31% (Figure 1C). Like the pattern observed with $_{V}$PLA, degradation leveled off after 48 h, leading to a total substrate hydrolysis of 36% (Figure 1E). Notably, Lip enhanced the release of LA from $_{PC}$PLA significantly by 9-fold at 48 h of incubation. However, a lower overall hydrolysis rate was observed compared with $_{V}$PLA, which can likely be attributed to its higher crystallinity, which limits substrate accessibility to the enzyme. Negative controls using BSA in place of Lip, as well as thermally inactivated Lip, resulted in a complete loss of synergistic activity toward $_{V}$PLA depolymerization (Supporting Information Figure S1).

Lipases are known to preferentially degrade PDLA,[30] while PrK exhibits hydrolytic activity exclusively toward PLLA.[31] To investigate whether the synergistic effect of Lip could be attributed to its ability to target and degrade portions of the substrate composed of the D-stereoisomer, the release of D-LA was quantified (Supporting Information Table S1). The latter revealed that only 2−5% of the total monomer released was in the D-conformation in every sample, indicating that the synergistic action of Lip is not driven by selective degradation of D-LA-containing regions of the substrate.

To further explore the role of Lip in its synergistic interaction with PrK, a test was conducted to characterize the hydrolytic activity of Lip toward oligo-lactides (O-LA). Currently, oligo-lactides (O-LA) are not commercially available, except for dilactide (DP2). Therefore, we first hydrolyzed PLA using PrK for 24 h and then removed the remaining insoluble film. The resulting supernatant, enriched in O-LA, was incubated with LiP for an additional 24 h (Figure 2A). This setup yielded different final concentrations of lactic acid (LA): 1.92 ± 0.05 g/L (equivalent to 15.1% of theoretical $_{V}$PLA conversion yield) with the PrK alone, and 2.83 ± 0.18 g/L (equivalent to 21.3% of theoretical $_{V}$PLA conversion yield) for the PrK followed by lipase treatment (Figure 2B). In comparison, a 24 h incubation with PrK yielded 1.1 g/L of LA. These results suggest that PrK releases a substantial amount of O-LA during the first 24 h of the reaction and that the Lip is active on the O-LA. To further investigate the lactides (O-LA), the supernatants from enzyme incubations were analyzed using high-resolution mass spectrometry (HRMS) (Figure 2C,D and Supporting Information Table S2). The PrK-treated sample exhibited a heterogeneous distribution of the oligomers of O-LA ranging from DP3 to at least DP11, with the highest abundance around DP4 (exact masses in Supporting Information Figure S2). In contrast, when the same supernatant was further incubated with Lip, a significant reduction in overall oligo content, approximately 30-fold lower ion counts per species, was observed. Particularly the longer oligomers, i.e., DP8−DP11, disappeared; exact masses and ion counts in Supporting Information Figure S3. This aligns with the increased concentration of monomeric lactic acid (LA) detected under the PrK + Lip condition compared to PrK alone.

Additionally, we evaluated the co-incubation of PrK and Lip in the presence of $_{V}$PLA, which resulted in a similar O-LA mass distribution and abundance as observed with PrK alone (Supporting Information Figure S4). Conversely, incubation with Lip alone did not yield detectable levels of O-LA, except for trilactic acid (Supporting Information Figure S5). These findings suggest that Lip can hydrolyze soluble O-LA only in the absence of the solid $_{V}$PLA film.

3.2. Synergistic Role of LOX I-B in PrK-Mediated PLA Degradation. A similar approach was adopted to investigate the effect of *G. max* lipoxygenase (LOX I-B) and its potential synergistic interaction with PrK in the hydrolysis of $_{V}$PLA and

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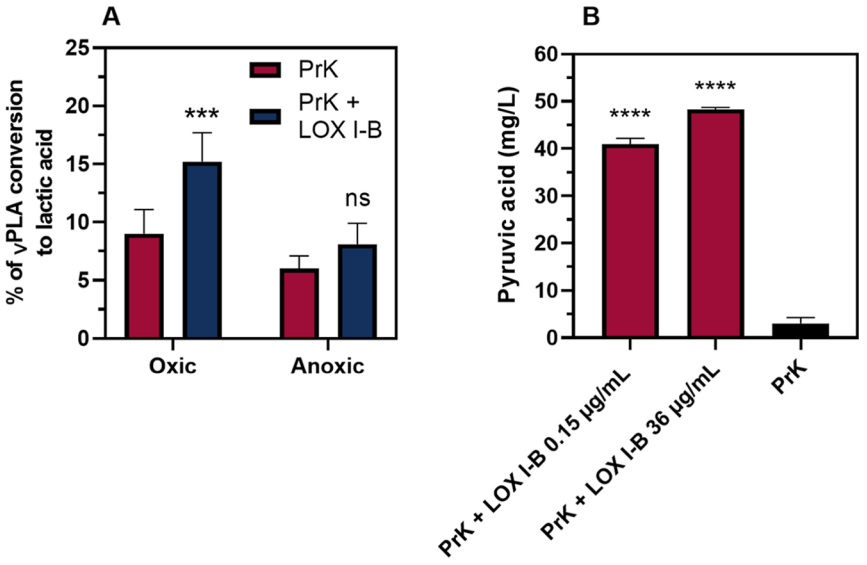

Figure 4. The effect of oxygen and lipoxygenase on PLA conversion to LA. (A) Synergistic effect of LOX I-B in combination with PrK on $_{V}$PLA tested in oxic and anoxic conditions. (B) The quantification of pyruvic acid by HPAEC chromatography. All reactions were carried out in 100 mM sodium phosphate buffer at pH 7.2, supplemented with 0.1 mM $CaCl_2$ and 1% (w/v) PLA film substrate. Enzyme concentrations were as follows: PrK at 0.43 mg/mL and LOX I-B at 0.15 μg/mL and 36 μg/mL. All data were collected in triplicate and synergism was tested using randomized two-way ANOVA.

$_{PC}$PLA. Oxidative reactions of PLA were conducted under the same experimental conditions described in the previous section in the presence of calcium to enhance the hydrolysis yield of PrK. LOX I-B is an iron-containing redox enzyme that catalyzes the oxidation of unsaturated fatty acids, to yield hydroperoxides. It has a well-established role in lipid metabolism but has never been investigated for its potential involvement in plastic oxidative degradation.

As presented in Figure 3A, LOX I-B alone showed no release of LA toward either $_{V}$PLA or $_{PC}$PLA, similarly to what was previously presented for Lip. However, when combined with PrK, LOX I-B significantly enhanced substrate degradation in a concentration-dependent manner, although not proportional. Specifically, a higher synergistic effect was observed on $_{V}$PLA, where LA release increased by 70% at lower LOX I-B concentrations and by 2.5-fold at higher concentrations. Although the effect was less pronounced for $_{PC}$PLA, it was still significant, with LA release increasing by 30% and 57% at low and high enzyme dosages, respectively. No synergistic effect on $_{V}$PLA depolymerization was observed with thermally inactivated LOX I-B (Supporting Information Figure S1).

Regarding the time course experiments presented in Figure 3B, it is possible to appreciate a typical non-Michaelis−Menten conversion kinetics for insoluble biopolymers, similar to lignocellulose derived fibers.[32] The contribution of LOX I-B in terms of lactic acid release by PrK from $_{V}$PLA remains constant for the entire course of the hydrolysis eventually reaching similar conversions obtained with a cocktail of PrK, Lip, and LOX-IB after 72 h (Figure 3C). Although the highest levels of $_{V}$PLA conversion into LA were those obtained by the mix of Lip with PrK, with conversion yield close to 60% of lactic acid production in 72 h (Figure 1D).

These degrees of synergism toward the two different kinds of PLA suggest an interaction of LOX I-B directly on the plastic polymer or the degradation of the oligomers rather than at the level of the soluble LA monomer, so ruling out a possible beneficial effect by the removal of possible product inhibition toward PrK.

3.3. LOX I-B Consumes Oxygen while Synergizing with PrK for the Degradation of PLA. Since LOX I-B functions as a dioxygenase, relying on molecular oxygen (O_2) to catalyze the oxidation of its substrates, the $_{V}$PLA degradation assay was repeated under anaerobic conditions to assess whether its enzymatic activity was indeed responsible for the observed synergistic effect. As shown in Figure 4A, the synergistic effect exerted by LOX I-B at the lowest dosage (0.15 μg/mL) decreased significantly by 53% under anoxic conditions, resulting in degradation levels comparable to those achieved by PrK alone. The loss of synergistic action underscores the enzyme's dependence on O_2 to enhance PrK-mediated $_{V}$PLA depolymerization, which suggests a catalytic activity of LOX I-B on the polymer or on the O-LA degradation products.

Two HPLC methods, one based on organic acid separation column (ROA H+) coupled with UV detection and a second based on anion exchange chromatography (HPAEC equipped with CarboPac PA1 column) coupled with an amperometric PAD detector, and HRMS spectrometry were used to identify possible oxidized products from PLA upon the catalysis of LOX I-B (Supporting Information Figure S6). A consistent presence of pyruvic acid (PA) was detected in the samples from incubation of $_{V}$PLA with LOX I-B in combination with PrK, respectively, 40.9 and 48.2 mg/L (respectively for the low and high dosage tested), corresponding to 0.3 and 0.4% of total PLA (Figure 4B). The incubation of the sole PrK on $_{V}$PLA instead resulted in 3 mg/L of PA, close to the detection limit of the HPAEC-PAD. No PA was detected for oxygenic incubation of LOX I-B alone with $_{V}$PLA.

Moreover, to elucidate further the possible presence of lactate oxidase enzymes in the commercial LOX preparation, or to test for activity of LOX I-B toward LA, direct oxidation of LA into PA was tested in either the presence or absence of $_{V}$PLA. No variation in the amount of LA before and after

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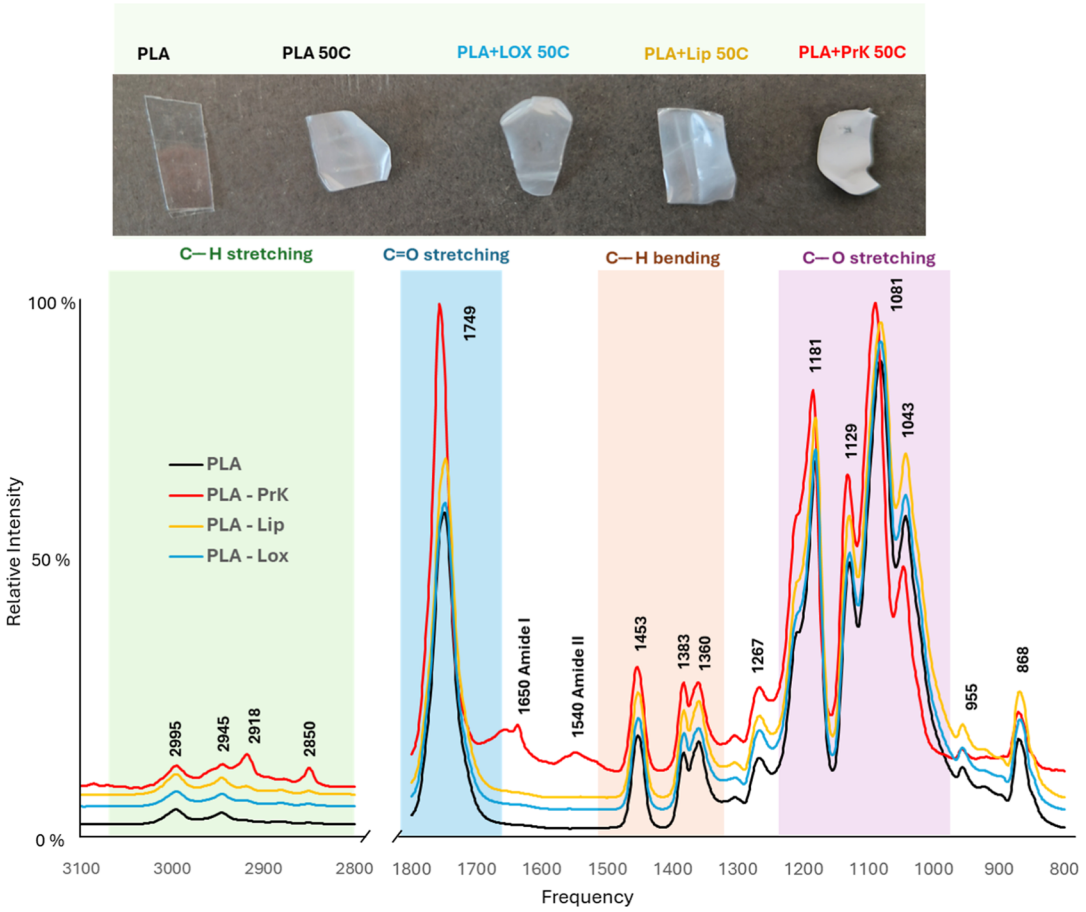

Figure 5. FTIR analysis of PLA materials upon incubation with various enzymes. (A) Pictures of $_{V}$PLA before and after enzymatic incubations. (B) FTIR spectra of $_{V}$PLA incubated at 50 °C (black line), with PrK (red), with Lip (yellow), and with LOX I-B (blue). Major regions of bond stretching and bending are highlighted.

incubation with LOX I-B could be observed, with the amount of LA that remained constant at 1 g/L as initially added, and no PA could be detected (Figure 7). Furthermore, lactate oxidases are known to produce hydrogen peroxide (H_2O_2) as a coproduct of the oxidative reaction of lactate to pyruvate, so the production of H_2O_2 was monitored using the Amplex Red method, which allows detection to the μM scale (Figure 8). Also, in this case, the amount of H_2O_2 produced when incubating LOX I-B (or PrK and Lip) with lactic acid or $_{V}$PLA, was below that of the control experiments lacking enzymes. Overall, the data presented here do not help in ascertaining if the LOX I-B preparation acts directly toward the PLA polymer or toward the O-LA degradation products derived from the simultaneous hydrolytic activity of PrK on PLA.

3.4. FTIR of $_{V}$PLA Incubated with Lipase, Lipoxygenases, and Proteinase K. The remaining pieces of $_{V}$PLA after 24 h incubation with all the individual enzymes presented a several grade of white opacification different from the totally transparent original material (Figure 5A). Surface and material structural changes after degradation were monitored using FTIR in Figure 5B, with highlighted single peak frequencies of the PrK sample that showed a slight blue shift compared to the other samples. The spectra presented a typical PLLA fingerprint with major peaks at 1745 cm^{-1} attributed to −C−O stretching vibrations from the ester, in the 1300 cm^{-1} to 1150 cm^{-1} region, having the C−O stretching modes (ester group) at 1265 cm^{-1} −C−O− bending and at 1182 cm^{-1} (−CO−C− stretching and ν O−C asymmetric mode at 1084 cm^{-1}). In this region, a major difference is noticed in the comparing of the sample incubated with PrK which presents a markedly reduced absorbance at 1043 cm^{-1} relative to C−CH_3 stretching motions, while the Lip enzymes present an increase in the same peak. These perhaps indicate an augmentation of crystallinity by Lip and relative amorphization due to the PrK enzyme. Consistently, the 921 cm^{-1} typical from a-crystal in PLLA[33] is observed for the sample with Lip and nontreated $_{V}$PLA but is absent in LOX I-B and the PrK sample.

The PrK sample also presented 2 peaks associated with the amide I and II at 1650 and 1540 cm^{-1}, respectively, indicative of enzyme absorption on the $_{V}$PLA surface; the same could not be observed for Lip and LOX enzymes, indicating a lack of detection below the instrument sensibility. This is perhaps due to the different dosages having the latter enzymes being several-fold lower compared to PrK. Overall, the $_{V}$PLA sample incubated with LOX I-B did not have signs of oxidation on the material surface.

3.5. Lipase and Lipoxygenases Promote Proteinase K Adsorption to the PLA Substrate. The enzymatic hydrolysis of polyester polymers occurs in three stages: complexation, catalysis, and dissociation. The plastic film represents a large surface area onto which the enzyme can adsorb. Once bound, the enzyme can displace a polymer chain and catalyze the reaction upon formation of a productive

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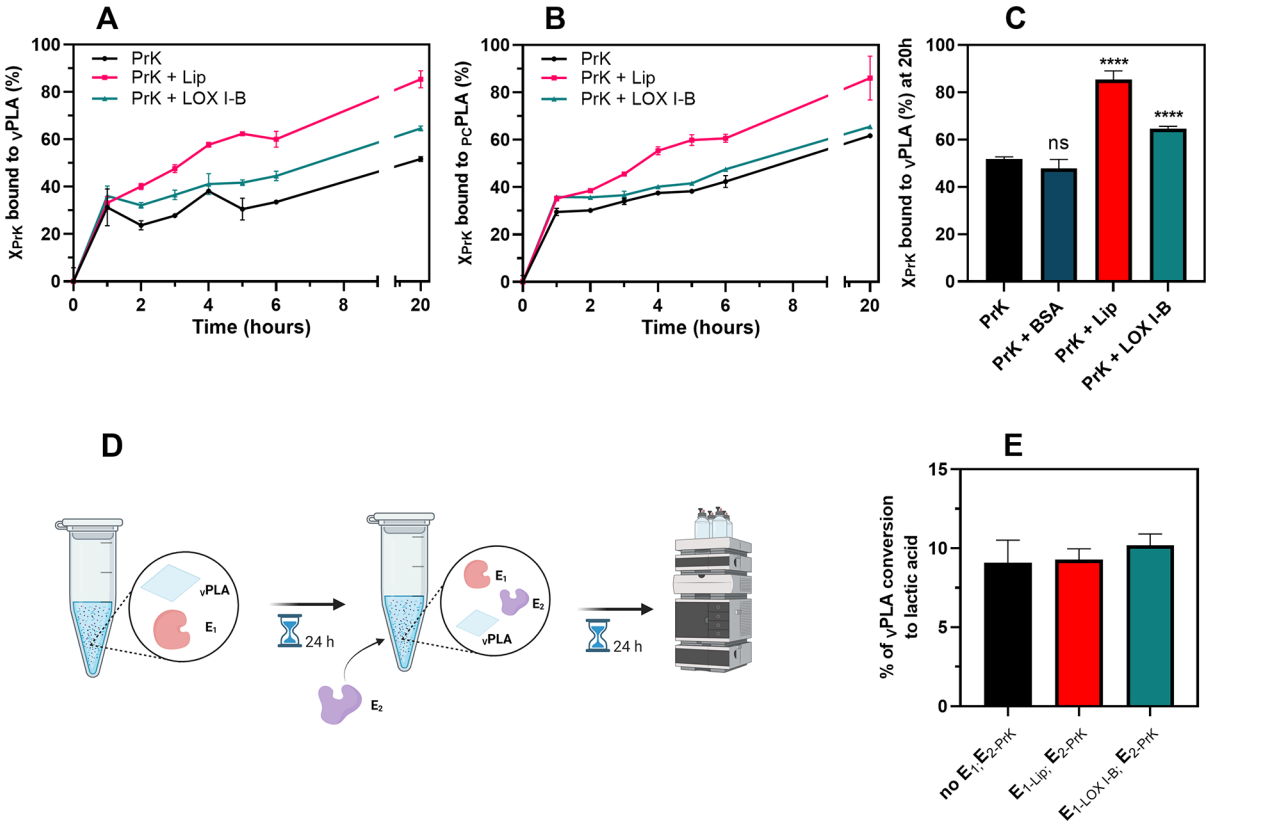

Figure 6. Adsorption assay and sequential addition of PrK, Lip, and LOX I-B. (A,B) PrK molar fraction (χ_{PrK}) bound to the substrate as a function of time during incubation on $_{V}$PLA (A) and $_{PC}$PLA (B), measured in the presence of Lip, LOX I-B, or PrK alone. Enzyme concentrations were as follows: PrK at 0.43 mg/mL, Lip 30 μg/mL, and LOX I-B 36 μg/mL (if not otherwise stated). The amounts of Lip and LOX in the mixtures do not significantly contribute to the quantified protein and can be regarded as negligible. (C) χ_{PrK} bound to the substrate at 20 h on $_{V}$PLA in the presence of Lip, LOX I-B, and a negative control BSA. (D) Schematic representation of the reaction setup for the sequential addition of the three enzymes, as in graph E. (E) Percentage of $_{V}$PLA conversion into lactic acid for the sequential addition of PrK, Lip, and LOX I-B experiment: E_1 represents the first enzyme added for 24 h and E_2 represents the addition of the second enzyme after 24 h.

enzyme−substrate complex. After the catalysis, the enzyme and products are released from the substrate, enabling the initiation of a new catalytic cycle.[34] To assess whether Lip and LOX I-B enhanced PrK-mediated PLA hydrolysis by increasing the number of productive binding sites (BSs) on the polymer, the free protein concentration was monitored during the reaction for PrK alone and in combination with Lip or LOX I-B, on either $_{V}$PLA (Figure 6A) or $_{PC}$PLA (Figure 6B).

After the first hour of the reaction, approximately one-third of PrK bound to the substrate with no major differences observed between the samples (Figure 6A,B). However, from the 2 h mark onward, significant differences emerged among the three conditions on $_{V}$PLA (Figure 6A). When PrK was used alone, its bound molar fraction (χ_{PrK}) remained relatively stable within the first 6 h, reaching a final bound fraction of 52%. In contrast, the addition of Lip significantly enhanced PrK binding to the polymer, with the bound fraction steadily increasing throughout the reaction, reaching 85% after 20 h. The addition of LOX I-B resulted in an intermediate effect. While it promoted PrK binding to the substrate, the increase was less pronounced compared to Lip, leading to an χ_{PrK} increase of approximately 33% more PrK bound to the polymer over the course of the reaction. The addition of bovine serum albumin (BSA) to PrK (Figure 6C) did not enhance PrK adsorption, showing no significant difference compared with PrK alone.

On $_{PC}$PLA, similar results were observed with the key difference being a reduction in the χ_{PrK} bound to the substrate in the presence of LOX I-B compared to that of PrK alone. The overall effect of LOX I-B was reduced to only a 10% increase in the χ_{PrK}. This finding aligns with the observed reduced synergistic effect mediated by LOX I-B on $_{PC}$PLA in terms of LA release (Figure 3A). Overall, these results indicate that Lip, and to a lesser extent LOX I-B, enhanced the binding efficiency of PrK. This effect is likely due to an increase in the number of BSs available for PrK on the substrate, ultimately leading to a higher depolymerization yield.

To further investigate the effect of Lip and LOX I-B on the polymer, a sequential enzyme addition was performed (Figure 6C). In this setup, the $_{V}$PLA film was first incubated with a Lip or LOX I-B (E1) and after 24 h a second enzyme (PrK, E2) was added, keeping the $_{V}$PLA film inside the test tubes. The data reported in Figure 6D are relative to the lactic acid measured at 48 h time-point instrumental to observe if one of the enzyme combinations could contribute to the hydrolysis during the course of the reaction of PrK. To our surprise, none of the enzymatic pretreatments with Lip or LOX I-B for 24 h led to an amelioration of the catalytic performance of the PrK in the successive 24 h. Overall, 1.3 and 1.2 g/L of lactic acid were measured for the Lip and LOX I-B pretreated $_{V}$PLA, while 1.2 g/L was obtained for the sample just hydrolyzed with PrK (without enzymatic pretreatment).

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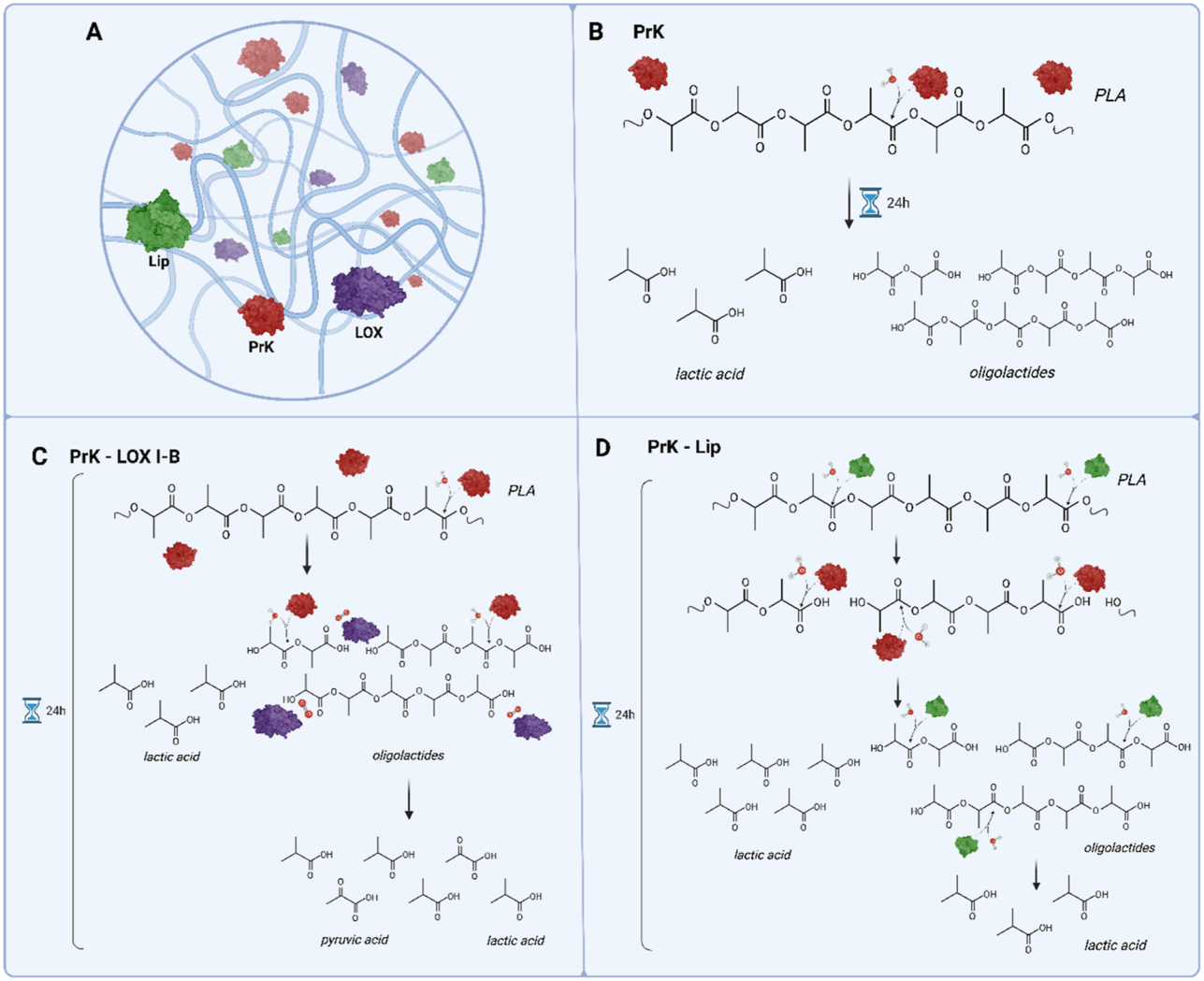

Figure 7. Model of synergism between PrK and LIP or LOX. (A) Schematic representation of polymer and enzymes: PrK (red), Lip (green), and LOX I-B (purple). Schematic representation of PLA degradation by PrK (B), PrK and LOX I-B (C), and PrK and Lip (D).

4. DISCUSSION

The present study investigated the synergistic interaction between Proteinase K (PrK), a well-established enzyme with PLA-degrading activity (Figure 7B), and two commercial enzymes, either hydrolytic *C. rugosa* lipase (CrLip) or redox *G. max* lipoxygenase (LOX I-B). Lip and LOX I-B did not generate a detectable release of monomers when incubated with two kinds of PLA (lab-casted and postconsumer); however, both enzymes significantly boosted PrK-mediated PLA degradation. Moreover, thermally inactivated Lip and LOX did not synergize with PrK, indicating that active enzymatic catalysis is required.

Lip enhanced PrK-mediated LA release by 5.7-fold on solvent-cast $_{V}$PLA films and 2.7-fold on $_{PC}$PLA films. Additionally, the presence of calcium further increased the overall hydrolysis yield, reaching 43.5% for $_{V}$PLA and 31% for $_{PC}$PLA within 24 h, in the presence of Lip and PrK. This increase in PLA hydrolysis aligns with previous reports indicating that PrK exhibits reduced thermal stability[35] and activity[29] in the absence of Ca^{2+}. However, PLA hydrolysis plateaued after 48 h, likely due to shear stress or autoproteolytic behavior of PrK, which becomes more pronounced at low enzyme concentrations.[36] This performance markedly exceeds that reported by Sourkouni et al. (2023),[37] who achieved only ~40% PLA weight loss after 6 weeks using a comparable dosage of a complex enzymatic cocktail composed of five enzymes, including both commercial lipases and proteases. In contrast, our system achieved approximately 60% degradation of $_{V}$PLA within only 72 h with a dosage of 0.05 g of enzymes per grams of the substrate. This highlights the superior efficiency of our approach, particularly when supplemented with Ca^{2+}, in accelerating PLA depolymerization under mild conditions.

Lip appears to enhance PrK activity through two potential mechanisms (Figure 7D): (i) increasing the number of productive binding sites for PrK on the polymer and perhaps (ii) hydrolyzing soluble O-LA oligomers released by PrK activity. The first hypothesis is supported by the observation that in the presence of Lip, the molar fraction of PrK adsorbed to the polymer increased by an average of 70% on $_{V}$PLA and 41% on $_{PC}$PLA compared to PrK alone, whereas the addition of a comparable concentration of BSA did not result in any increase in adsorption. This suggests that Lip may function as a PLA-modifying noncatalytic enzyme, rather than a PLA-degrading enzyme,[38] altering the polymer surface to enhance PrK binding. This hypothesis is further reinforced by previous studies showing that while Lip did not release LA from PLA,[28]

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it could still induce surface modifications, such as small cracks in nonwoven PLA fibers.[26,39] However, if added to the same previous setting after 24 h of PrK action with the latter still present and the $_{V}$PLA film, the Lip did not synergize with PrK. It appears that the synergistic activity between these two hydrolytic enzymes may lie in the promotion of PrK binding to the PLA film and the partial removal of the generated PLA-oligomers (Figure 7).

Similarly, LOX I-B exerted a significant synergistic effect when combined with PrK, resulting in a 2.5-fold increase in $_{V}$PLA hydrolysis and 57% in $_{PC}$PLA when used at a dosage similar to that of Lip. The observed synergism can be attributed to the increased χ_{PrK} bound to the substrate in the presence of LOX I-B, although not to the same extent as Lip. This effect was particularly pronounced for $_{V}$PLA, where LOX I-B led to a greater enhancement of the hydrolysis yield. This suggests that LOX I-B also enhanced the PrK activity by facilitating its adsorption onto the polymer surface. However, the FTIR analysis did not detect signs of surface oxidation on the $_{V}$PLA. Moreover, in support of the hypothesis that LOX I-B plays a catalytic role in the depolymerization of PLA, the removal of molecular oxygen resulted in a significant loss of synergism with PrK. This O_2-dependence provides further evidence that LOX I-B actively participates in the catalysis of PLA, and the amount of pyruvate was detected as a result of their incubations with $_{V}$PLA. However, LOX I-B is a 13-lipoxygenase, which specifically oxidizes the C13 position of linoleic acid within *cis*,*cis*-1,4-pentadiene moieties, which are lacking in PLA. Nevertheless, this represents only the primary reaction catalyzed in vivo, as LOXs are also known to facilitate the subsequent conversion of hydroperoxides into various secondary oxidation products[40] and to mediate nonspecific oxidative reactions. For instance, Enoki et al. (2003)[41] reported a reduction in the molecular weight of *trans*-1,4-polyisoprene following incubation with LOX and its oxidative mediator, linoleic acid. More recently, Gianni et al. (2018)[42] documented LOX-mediated oxidation and fragmentation of lignin via oxygen- and carbon-centered radicals, both in the presence and absence of linoleic acid working as a mediator. Given the known oxidative capabilities of LOX I-B, its potential activity toward PLA may involve the initiation of radical-mediated reactions (Figure 7C). LOX I-B could facilitate the formation of reactive oxygen species or radical intermediates on the PLA-oligomers (O-LA), which may, in turn, interact with the polymer backbone. This could lead to oxidative modifications, such as chain scission or cross-linking, depending on the reaction conditions and the nature of the formed radicals. In support of such controlled radical shuttling from O-LA to PLA, no signs of H_2O_2 were detected during incubation of LOX I-B with and without $_{V}$PLA.

Overall, both synergistic actions described in this work obtained with minimal addition of auxiliary enzymes (Lip and LOX I-B dosed at 2% with the respect to PrK) represent a promising step forward for scalability of the PLA biodegradation.[43]

5. CONCLUSIONS

Lipoxygenase and lipase enzymes synergize with proteinase-K during degradation of PLA enzymes when incubated simultaneously. The synergism resulted in up to 3- and 4-fold increase of PLA conversion to lactic acid. We investigated the profile of products released during each reactions with HRMS and revealed that *T. album* PrK release a conspicuous amount of Oligo-LA that are recognized as substrates by the *C. rugosa* lipases hydrolyzing them into lactic acid. Also, we could measure amounts of pyruvic acid as an oxidation product of PLA chains during oxygenic incubation with lipoxygenases. We hypothesize that *G. max* LOX-IB can oxidize the oligo-PLA which transfer the carbon-centered radical to the PLA film in a mediator style.

This study did not aim to optimize enzyme ratios or reaction conditions for maximal PLA degradation; however, it demonstrated that the combined action of PrK and Lip or LOX I-B, despite their low molar concentration (i.e., approximately 2% of that of PrK) substantially improved PLA degradation with a remarkable synergy of ∼60% hydrolysis of amorphous PLA in 72 h. This finding is particularly relevant for future research, as leveraging such synergism could allow for a reduction in the overall enzyme loading required for effective PLA depolymerization. By enhancement of degradation efficiency with minimal additional catalyst input, this approach represents a promising step toward a more cost-effective and scalable enzymatic recycling strategy for plastic waste.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.5c06901.

HRMS analysis of oligo-lactides, HPAEC detection of pyruvic acid, quantifications of D-lactic acid, activity of LOX I-B on lactic acid, ROS detection, and control experiment with inactivated enzymes and BSA (PDF)

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Notes

The authors declare no competing financial interest.

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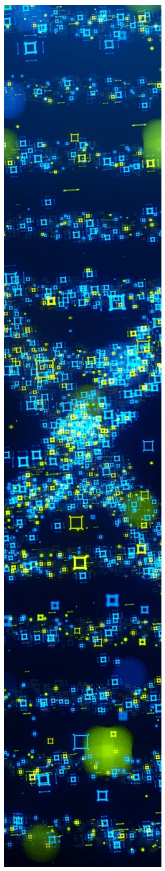
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