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Dedicated to Niculae I. Ionescu PhD
on the occasion of his 85th anniversary

THERMAL ANALYSIS FOR DEPICTION OF A LACCASE-PVA BIOCOMPOSITE

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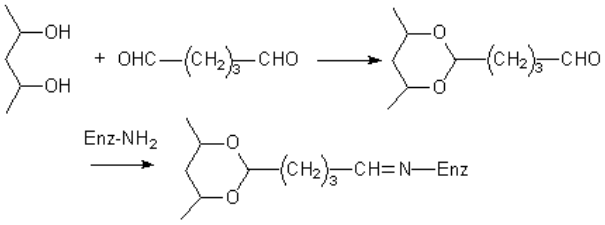
The characterization, by thermal analysis, of a biocomposite material has been performed. The biocomposite was obtained by the covalent immobilization of a commercial laccase on a cryogel PVA [Poly(vinyl alcohol]. Its thermal behaviour was compared with those of its components: the commercial enzyme (containing additives for stabilization), and the polymer carrier, as well as an enzyme free of additives. The biocomposite presents a higher thermal stability compared with those of its components. Thus, the immobilization leads to the enhancement of the thermal stability for the enzyme and the carrier. The carrier behaviour was discussed in comparison with literature data on PVA. The previous functionalization of the carrier, by treatment with glutaric aldehyde, generates a reticulated structure, enhancing the polymer stability. According to the experimental results, the immobilization process leads also to the purification of the enzyme, by reducing considerably the content of commercial enzyme additives into the biocomposite.



INTRODUCTION

The limited stability of enzymes restricts their industrial large scale applications. Different approaches have been used for stabilizing enzymes, like: immobilization, protein modification or reaction environment modification. Immobilized enzymes, considered bio-based composites, are of great interest for the progress of different industrial fields.[1-3] Enzyme immobilization was frequently used for stabilization of enzymes.[4-12] By immobilization a number of improvements of enzyme properties are performed, like:

- higher stability;
- easy separation of the biocatalyst from the reaction mixture;
- reuse of the biocatalyst through a number of cycles;
- changes for some enzyme characteristics resulting better yields (*e.g.* **optimal pH** change leading to better reaction conditions for the reagents and the products; **temperature stability** changed by raising it and consequently enhancing reaction yield, etc.).

Laccases are a family of oxido-reductases used in textile and food industry for product quality

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enhancement or for waste water treatment. Some recent review papers described such laccase applications in different fields (food, textile, de-pollution, organic synthesis, etc.) at laboratory or even industrial scale.[13-20] In a number of applications, immobilized laccases have been successfully used.[21-24] The immobilization process may be performed by using different methods (covalent bond, enzyme cross-linking, adsorption or entrapment), each one with advantages and disadvantages. The covalent method for immobilization of the enzyme enhances its stability, avoids enzyme leakage from the bio-composite, but may reduce the activity, due to conformational modifications and diffusion problems.[5] A covalent immobilization of a commercial laccase has been previously performed, using a wide-pore cryogel type Polyvinylalcohol (PVA) as carrier.[25] The immobilization was performed using previously glutaraldehyde for the polymer carrier functionalization.

The thermal behaviour of the new biocomposite has been studied and compared with those of its components: the cryogel polymer, the functionalized polymer, as well as with literature data for the habitual PVA polymer. The thermal behaviour of commercial laccase was also compared with that of a pure enzyme sample supplied by Sigma. The obtained data may give information about the composition, as well as the stability of the studied biocatalyst.

RESULTS AND DISCUSSION

The studied biocomposite has as carrier PVA, one of the most produced polymers.[26] It has numerous applications due to its biodegradability which makes this polymer environmentally safe.[27] The fabrication procedure, by freezing-thawing processes, generates a wide-pore polymer suitable for enzyme immobilization.[28]

The covalently immobilized enzyme was synthesized by a multi step process[25] (Scheme 1). The carrier was previously functionalized by treatment with glutaric aldehyde, when cyclic acetals, of the vicinal OH groups of the polymer, are formed. The commercial laccase was then immobilized by condensation of the free amino groups of the enzyme protein with the free carbonyl groups of the functionalized polymer.

The thermal behaviour of enzymes is of great interest, information concerning the protein folding or stability may be obtained by analyzing the experimental data.[29,30] Thus, the study of the thermal stability of the covalently immobilized laccase was performed and compared with those of its components the enzyme and the carrier. As a general pattern, the behaviour of all the analyzed samples is not different in air and nitrogen, proving that the oxidation reactions are not the main route for decomposition. The copper from the catalytic center gives the residual part.

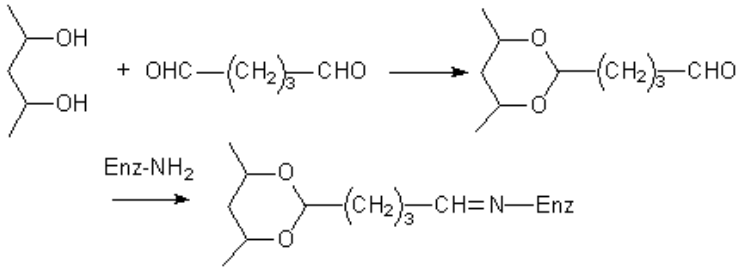
The pure and commercial enzymes thermo-gravimetric registered curves are presented in figures 1 and 2.

The thermo-gravimetric curve of the free enzyme (see Figure 1) evidenced two steps in the process of the pure enzyme decomposition. Most probably, in the first step, at 286.4 ^{0}C (**a**) or 288.4 ^{0}C (**b**), the glycosylated chains of laccase protein are decomposed, and the functional groups of the component amino acids are eliminated as: H_2O, CO_2, etc. The second transformation happened at 382.0 ^{0}C (**a**) or 382.7 ^{0}C (**b**), being most probably a fragmentation of the protein polyamide chain.

For the commercial enzyme (see Figure 2) the additive decomposition pattern is observed at 214-229 ^{0}C. The peaks showed clearly the high amount of stabilizing agents (mostly adipic acid[31]) in respect with the enzyme content in the commercial product (Mass loss of 3.8% representing the protein+ glycosyl groups). This result is confirmed by the analysis of protein content, previously performed. According to this analysis the protein content is of 2.57 ± 0.2 % (average value of triplicate experimental determinations).[32] It is worthwhile mentioning that the thermal analysis may evidence the solid mix composition as previously ascribed for a polymer mixture.[33] Complementarily, the stabilization of the commercial laccase, compared with the pure enzyme, is illustrated by the displacement of the first decomposition peak of the enzyme to a higher temperature value (~344 ^{0}C *vs.* ~289 ^{0}C, see Figures 1 and 2).

For a clear picture concerning the immobilized enzyme the decomposition pattern of the PVA polymer carrier has to be also studied. The analysis of the thermal behaviour of the cryogel PVA gives information concerning its stability. A very complex pattern is obtained for both the cryogel PVA and the functionalized cryogel polymer. It is noticeable that the cryogel PVA is more stable, decomposing at higher temperature (~291^{0}C, see Figure 3) than the classical PVA polymer (~250^{0}C).[34,35] The main route for the

decomposition process, previously described in literature,[35] consists in the loss of water with formation of double bonds in the polymer chain. The higher value for the decomposition temperature for the cryogel carrier reflects a higher stability of the polymer due most probably to formation of a hydrogen bond network, through the freezing-thawing process of synthesis.



Scheme 1

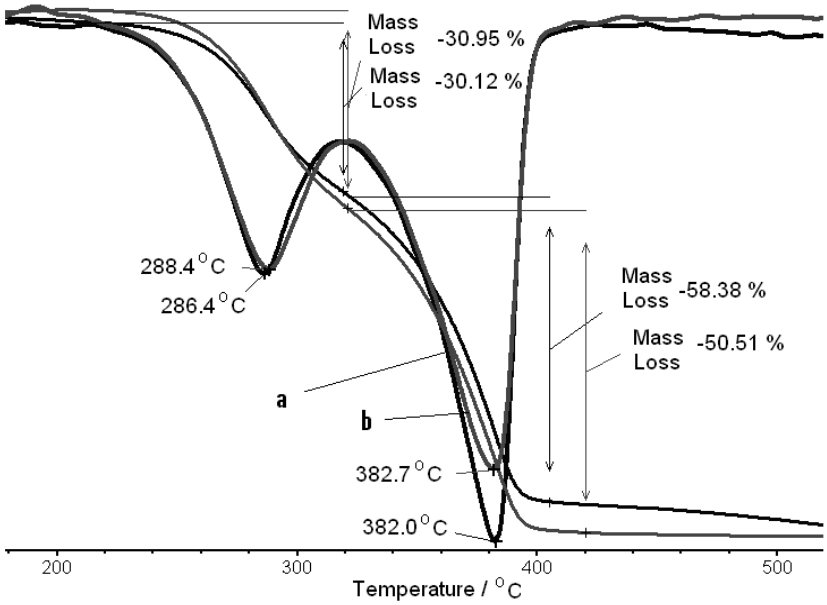


Fig. 1 – Pure laccase thermogravimetric curves registered in air (**a**) and nitrogen flow (**b**).

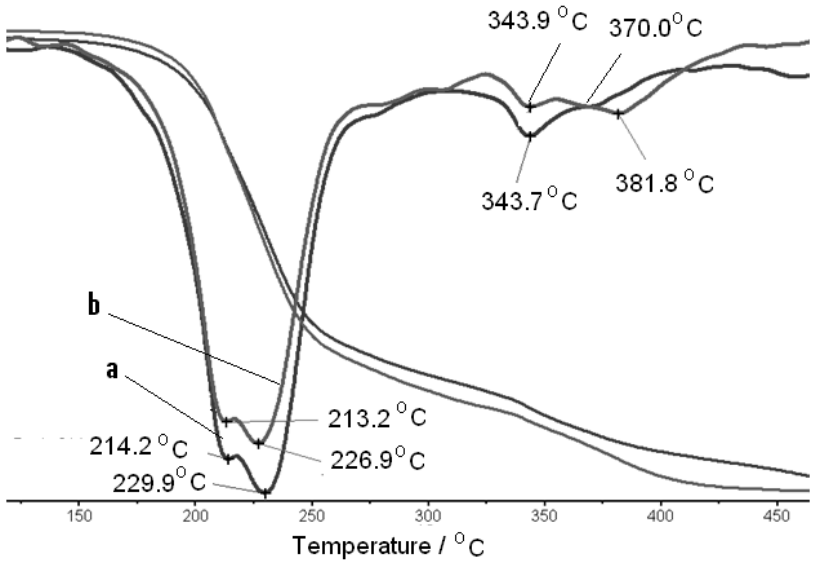


Fig. 2 – Commercial laccase thermogravimetric curves registered in air (**a**) and nitrogen flow (**b**).

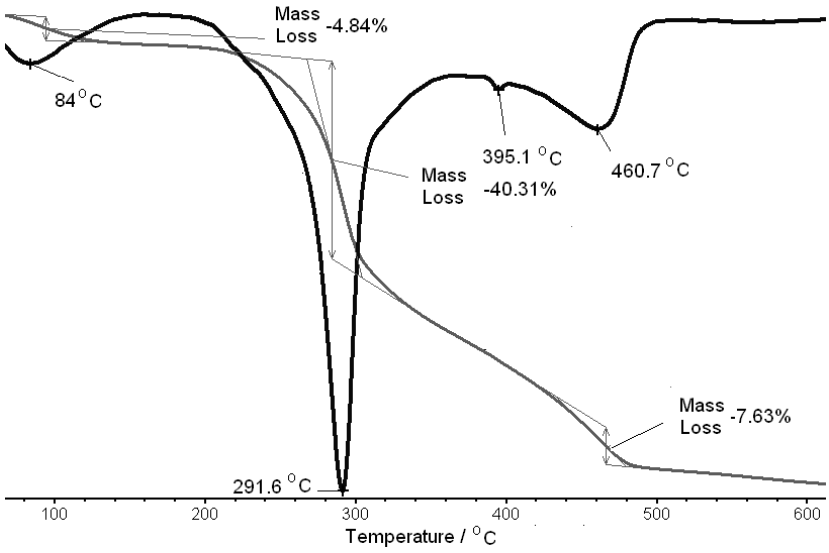


Fig. 3 – Thermal decomposition of cryogel PVA in air flow.

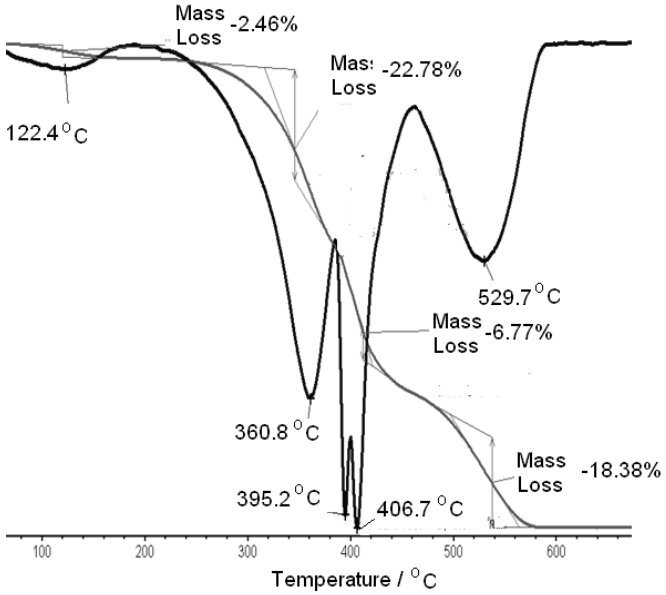


Fig. 4 – Thermal decomposition of functionalized cryogel PVA in air flow.

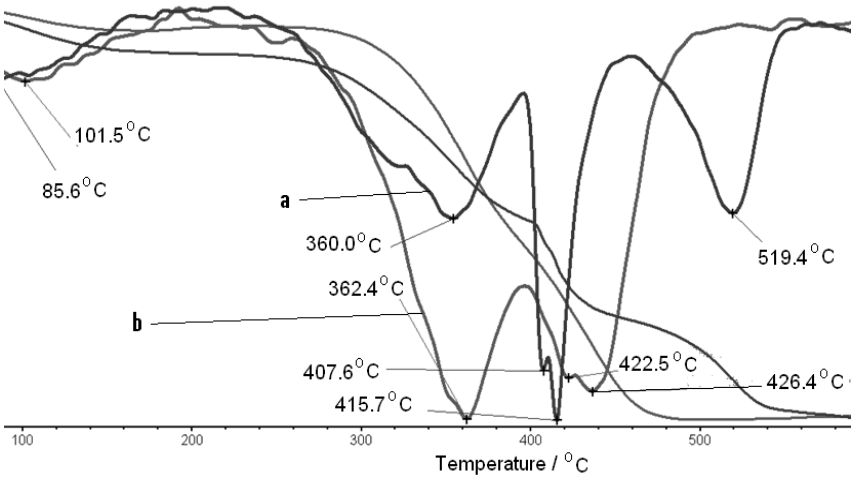


Fig. 5 – Immobilized laccase thermogravimetric curves registered in air flow (a) and in nitrogen flow (b).

The transformation of -OH groups on treatment with glutaraldehyde moves the decomposition process to even higher temperature (~361^{0}C) leading to a more complex aspect (see Figure 4). Similar observations have been evidenced during other functional modifications done to the PVA polymer.[36]

A very complex pattern was obtained at the decomposition of the immobilized enzyme (see Figure 5). By comparing the curves characteristics for the commercial enzyme, the polymer carrier and the bio-composite, the following assertions resulted:

- The general shape of the thermogravimetric curves is similar for the polymer and the immobilized enzyme biocomposite;
- The enzyme presence is better evidenced by the analysis performed in air by a shoulder at 360^{0}C, a value higher than in the case of the commercial enzyme 344^{0}C, indicating a stabilization by immobilization;
- In the case of nitrogen flow decomposition the enzyme presence is evidenced by the enhancement of the first peak of the carrier, both peaks for enzyme and carrier covering the same area of temperatures;
- The additives pattern is not observed in the biocomposite, the immobilization in the applied experimental conditions leading also to a purification of the enzyme.

MATERIAL AND METHODS

A pure sample of laccase was purchased from Sigma. The commercial enzyme was supplied by Rotta. The polymer carrier was prepared by the research group of Prof. Lozinsky. The PVA cryogel was prepared by cryotropic gelation, which takes place on moderate freezing followed by storing in a frozen state and subsequent thawing of solutions containing the polymer precursors.[37] A macroporous polymer was obtained with visco-elastic properties providing good resistance to abrasion and high elasticity.[38]

For immobilization the carrier was previously functionalized, by treatment with glutaraldehyde, and then the laccase was linked by the condensation of the amino groups of the enzyme protein with the free carbonyl groups of the carrier, with formation of imino-bound (see Scheme 1). The details concerning the preparation (ratio between reagents, reaction conditions, etc.) have been described in a previous work[25] (see procedure a).

The thermogravimetric experiments, with determination of the TG/DTG variation, have been performed on a Netzsch Luxx STA system between 30^{0}C and 600^{0}C using Al_2O_3 crucibles. The thermal investigations were performed alternatively in air/nitrogen flow (50 mLmin^{-1}) at a 5 Kmin^{-1} heating rate, for samples previously dried at the ambient temperature. The experimental mass samples have been of 5mg.

CONCLUSIONS

The thermal analysis of the immobilized enzyme, through comparison with the thermal behaviour of its components, offers a number of information about the biocatalyst. Based on these analyses the following statements may be formulated:

The decomposition environment (air or nitrogen) does not influence too much the pattern of thermal degradation for the biocomposite, as well as for its components.

The cryogel-type PVA is thermally more stable compared to a classic PVA.

The functionalization by treatment with glutaraldehyde stabilized further on the PVA carrier.

The commercial enzyme is thermally more stable due to the additives (main component adipic acid) than the pure product.

The biocomposite presents a complex decomposition process, influenced mainly by the pattern of the carrier due to the low enzyme content.

According to the experimental results the commercial enzyme additives are no more present into the biocomposite, immobilization leading to enzyme purification.

In the immobilized enzyme, both carrier and enzyme have increased in some respect the decomposition temperature, confirming a further stabilization by immobilization of both carrier and enzyme.

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