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Covalent Immobilization of α-Amylase onto Thermally Crosslinked Electrospun PVA/PAA Nanofibrous Hybrid Membranes

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ABSTRACT: Poly(vinyl alcohol)/poly(acrylic acid) (PVA/PAA) nanofibers with the fiber diameter of 100–150 nanometers were fabricated by electrospinning. PVA/PAA nanofibers were crosslinked by heat-induced esterification and resulting nanofiber mats insoluble in water. α-Amylase was covalently immobilized onto the PVA/PAA nanofiber surfaces via the activation of amine groups in the presence of 1,1′-carbonyldiimidazole. The immobilized α-amylase has more resistance to temperature inactivation than that of the free form and showed maximum activity at 50°C. pH-dependent activities of the free and immobilized enzymes were also investigated, and it was found that the pH of maximum activity for the free enzyme was 6.5, while for the optimal pH of the immobilized enzyme was 6.0. Reuse studies demonstrated that the immobilized enzyme could reuse 15 times while retaining 81.7% of its activity. Free enzyme lost its activity completely within 15 days. Immobilized enzyme lost only 17.1% of its activity in 30 days. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: nanofiber; PVA/PAA; amylase; immobilization; electrospun

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INTRODUCTION

Amylases are enzymes which hydrolyze starch molecules to give diverse products including dextrin and progressively smaller polymers composed of glucose units. They are one of the most important and oldest industrial enzymes for all starch-based industries and classified as α- and β- amylases according to the anomeric type of sugars produced by the enzyme reaction. α-Amylase (1,4-α-d-glucan-glucanhydrolase, EC. 3.2.1.1) is a universally distributed secretary enzyme throughout the animal, plant and microbial kingdoms.[1]

Biotechnology is currently considered as a useful alternate to conventional process technology in industrial and analytical fields. The demand for industrial enzymes is ever increasing owing to their applications in a wide variety of processes. Immobilized enzymes are preferred over the native ones as they can offer many advantages over their free forms, which include easy recovery and possible reuse, improved stability, and avoiding protein contamination of the final product.[2]

There are various methods of immobilization of enzymes on many different types of supports. There have been many reports about immobilization of α-amylase used for the hydrolysis of starch and production of maltose. Each immobilization method has its own advantages and disadvantages as immobilization is generally accompanied by changes in enzymatic activity, optimum pH, temperature, and stability.[3,4]

Synthetic polymers are widely used as enzyme immobilization carriers because they show perfect mechanical stability; they are less susceptible to bacterial attacks and easily prepared in desired geometrical configurations.[5] To achieve high enzyme loading and catalytic efficiency for large-scale operation and application, supports with high surface to volume ratio are often desirable. Therefore, in recent years, nanostructured supports such as mesoporous materials, nanoparticles, and nanofibers have been widely studied for enzyme immobilization.[6]

Electrospinning is a simple and versatile method for producing nanofibers from a variety of materials with fiber diameters ranging from several micrometers down to tens of nanometers. The collected nanofiber mat shows many exciting characteristics such as large surface area to volume ratio, good mechanical strength, excellent flexibility, and high porosity.[7] The electrospun nanofibers have a high specific area, so they are excellent candidates for filtration, drug delivery carrier, tissue engineering, wound dressing, nanosensors, and enzyme immobilization.[8]

Poly(vinyl alcohol) (PVA) is a nontoxic, biologically compatible, water soluble low-cost synthetic polymer with good chemical and thermal stability. PVA has been widely used in a wide range

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of applications in medical, cosmetic, food, pharmaceutical and packaging industries, as well as for cell and enzyme immobilization.[9] Poly(vinyl alcohol)/poly(acrylic acid) (PVA/PAA) is a typical blend system in which molecular level miscibility is achieved by interpolymer hydrogen-bonding interactions, and it was assumed that the crosslinking of the completely miscible blend system occurs via dehydration between carboxylic acid and hydroxyl group, that is, esterification and strong bonding during thermal activated reaction.[10]

The process described in this work is a convenient approach to fabricate thermally crosslinked PVA/PAA nanofibers with reactive groups for covalent enzyme immobilization. Herein, we report here for the first time covalent immobilization of α-amylase onto the thermally crosslinked electrospun PVA/PAA nanofibers. The optimum pH and temperature for starch hydrolysis as well as the kinetic parameters, thermal stability, reusability, and storage stability of enzyme were studied under free and immobilized conditions.

EXPERIMENTAL

Materials

PVA (molecular weight = 146,000–186,000, 87–89% hydrolyzed), PAA (molecular weight~ 450,000), α-amylase (1,4-alpha -D-Glucan-glucanohydrolase; EC 3.2.1.1, Type VI-B from the porcine pancreas, extra pure 35 U/mg), starch, maltose, 3,5-dinitrosalicylic acid (DNSA), and bovine serum albumin (BSA) were all obtained from Sigma Chem. Co (St Louis, MO). Coomassie-Brilliant Blue G-250 and 1,1′-carbonyldiimidazole (CDI) was purchased from Fluka AG (Switzerland). All other chemicals were of analytical grade and were purchased from Merck AG (Germany).

Preparation of Electrospun PVA/PAA Nanofibers

PVA was dissolved in deionized water and gently stirred for 2 h at 80°C to prepare 5% w/w solution. PAA was dissolved in deionized water and stirred for 4 h at room temperature to have 10% w/w concentration. Aqueous solutions of PVA and PAA were mixed with 10:1 weight ratio under stirring at room temperature.

Nano FMG NE100 lab scale electrospinning unit was used for electrospining. Ten milliliter of PVA/PAA mixture was loaded into a syringe fitted with a stainless steel needle and attached to a power supply and electrospun into nanofiber web under following conditions: voltage: 25 kV, syringe rate: 1 mL/h, and tip to collector distance: 15 cm. Electrospun nanofibrous mats were carefully detached from the collector and thermally crosslinked at 140°C.[11]

Activation of PVA/PAA Hybrid Nanofiber Mats

The hydroxyl group of the PVA/PAA was reacted with CDI, and the final product has an amine reactive imidazolyl-carbamate group. 6.5 g crosslinked PVA/PAA nanofiber mat was transferred into a three-neck round-bottomed flask filled and purged with nitrogen atmosphere. Ten grams (0,062 mol) CDI in 50-mL dry THF was added to the reaction flask and stirred magnetically for 24 h. The reaction temperature was 40°C. THF was distilled off in a rotary evaporator, and the activated PVA/PAA was stored at 4°C until use.[12]

Enzyme Immobilization

Amine reactive PVA/PAA nanofiber mat was added to 1.1 g (38,500 U) α-amylase in 250-mL 10-mM phosphate buffer (pH 6.9). Immobilization process was performed over 12 h at 25°C with constant shaking in a water bath (Clifton Nickel-Electro, Digital Shaker Bath, Labor Teknik, Istanbul, Turkey). Resulting amylase-immobilized nanofiber mat was filtered and unbound enzyme was removed by washing the support three times with 10-mM phosphate buffer. The washing solution was kept for measuring the amount of covalently bound enzyme on the support. The enzyme protein concentration within the extract was measured using Coomassie Brillant Blue reagent as described by Bradford.[13] A calibration curve prepared with BSA solution of known concentration was used in the calculation of protein in the enzyme and wash solution. From the results of protein recovery, the amount of immobilized enzyme per weight of material was calculated.[12]

Effects on Enzyme Activity

The activities of free and immobilized α-amylase were determined by the assay suggested by Bernfield.[14] Briefly, 1% w/w starch solution was prepared by dissolving soluble starch in 100-mL 10-mM phosphate buffer (pH 6.9). A known amount of hybrid nanofiber containing immobilized α-amylase was placed into a test vial. Then, 1-mL starch solution was added, and the system was incubated in a water bath with constant shaking at 30°C for exactly 5 min. The reaction was terminated by adding 1 mL of DNSA reagent after nanofiber mat taken out from the vial. Incubation was performed in a boiling water bath for 5 min. The amount of reduced sugar (maltose) produced was determined spectrophotometrically at 540 nm. In each set of experiments, a standard curve was prepared with maltose solutions of different concentrations. An enzyme activity unit (IU) was defined as the amount of enzyme liberating 1-μmol maltose per minute under the assay conditions. Each determination was carried out in triplicate. Activity of the enzyme is calculated from the following equation:

$$\text{Activity}\left(\text{IUmg}^{-1}\right) = \frac{\text{releasedmaltose}(\mu\text{mol})}{\text{amountof}\,\alpha - \text{amylase(mg)} \times \text{min}}$$

The effect of temperature on enzyme activity in the range of 30–80°C and the effect of pH on enzyme activity in the range of pH 5.0–8.0 was also studied.

Kinetics of Free and Immobilized Amylase

For the determination of kinetic constants, the initial rates of the reaction of the enzyme with starch solution using different concentration of the substrate were determined (0.25–2.5 mg/mL).

Reusability of Immobilized Amylase

One milliliter of 1% w/w starch in 10 mM phosphate buffer (pH 6.0) was added to the immobilized enzyme and incubated for 5 min at 50°C under constant shaking for each cycle. At the end of the reaction, immobilized enzyme was taken and washed with distilled water and then added a substrate solution to start a new cycle. The supernatant was assayed for reducing sugars.[12]

Effect of Storage Time of Immobilized Amylase

The storage time of immobilized α-amylase was determined by carrying out the pH 6.0 and 50°C at different times (1–30

days). The residual activities were calculated as percentage of the initial activity.

Characterization of Nanofibers

The functional groups of PVA/PAA nanofibers and enzyme immobilized nanofibers samples were obtained with Perkin-Elmer (ATR-FTIR) spectrometer. The nanofiber morphology was determined using scanning electron microscopy (SEM) (Phillips XL 30 ESEM-FEG). The average diameter and the diameter distribution were obtained by using a custom code image analysis program to analyze the SEM images. A small section of the nanofiber mat was placed on the SEM sample holder and sputter coated with gold. The majority of SEM images were taken at 1000×, 5000×, and 20,000× magnifications. Elemental concentrations for carbon, nitrogen, and oxygen were performed with an energy dispersion spectrometer (EDS) OXFORD INSTRUMENTS-INCA, Model No. 7274.

RESULTS AND DISCUSSION

Fabrication and Characterization of the PVA-PAA Nanofibrous Hybrid Membranes

In this work, for the first time, we immobilized α-amylase onto PVA-PAA nanofibrous hybrid membranes. PVA/PAA hybrid nanofibers generated from the mixtures of PVA/PAA at 10:1 weight ratios by using heat-induced crosslinking. Upon heating at 140°C, all membranes became insoluble in water, giving evidence of thermally induced esterification reaction between PAA and PVA. To covalently immobilizing enzyme on these membranes, a two-step process was used. First, the free hydroxyl groups on the PVA-PAA nanofiber membrane surfaces were activated with CDI. Second, the condensation reaction of the amino groups of enzyme with the activated imidazolyl-carbamate groups was carried out. During the reaction, amide bonds are formed between the amino groups of the enzyme and the imidazolyl-carbamate groups of the membranes. Figure 1 shows schematic representation of activation and immobilization of enzyme on nanofiber membrane.

Figure 2 shows the FTIR spectrum of neat and amylase immobilized PVA/PAA nanofiber mats. It can be seen that the enzyme-immobilized nanofibers show new IR absorption bands at 1555 cm^{-1} (amide II) and 1640 cm^{-1} (amide I). In general, the amide I and amide II bands appear as a result of the stretching vibration of the C=O bonds and a combination of C—N stretching and N—H bending vibrations in the backbone of the enzyme, respectively.[15] FTIR results show the successful immobilization of amylase onto the PVA/PAA nanofiber.

The morphology of the PVA-PAA nanofiber mat before and after enzyme immobilization was observed by using the SEM. As shown in Figure 3(a), an almost homogenous network of the electrospun nanofibers with diameters in the range of 100–200 nm was obtained when the applied voltage was 25 kV. The nanofibers show a smooth surface and a uniform body with a narrow distribution of fiber diameter.

Figure 3(b, c) shows the SEM micrographs of amylase immobilized nanofibers. It was observed that the enzyme molecules were covalently bonded on nanofiber surface. The covalent bonding between nanofiber surface and enzyme changes fiber

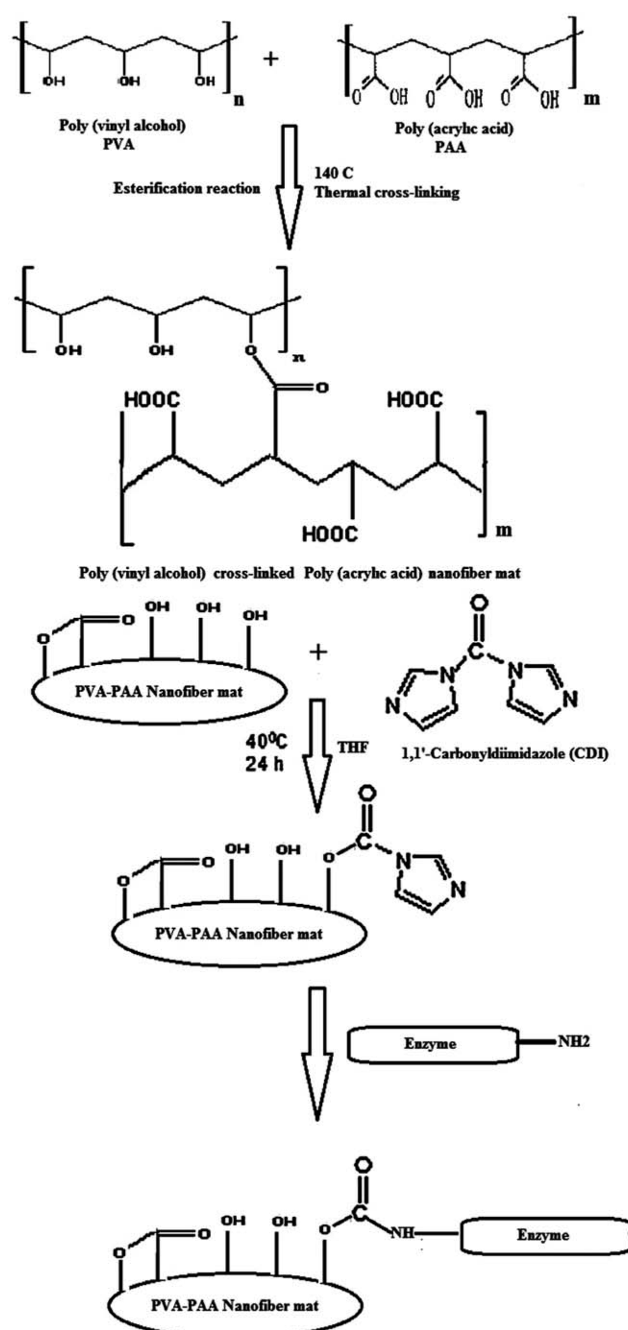

Figure 1. Thermal crosslinking mechanism between PAA and PVA via esterification reaction and schematic representation of the covalent attachment of enzyme.

morphology and as a result, the nanofiber morphology is distinctly different as compared to the morphology of nanofiber before immobilization.

To determine the chemical composition of this ultrastructure SEM-EDS was performed. The composition of the prominent included carbon (63.08%), nitrogen (7.83%), oxygen (27.76%), and sodium (1.33%). The unmodified nanofiber support possesses carbon and oxygen atoms in their structure. However, the nitrogen atoms can be introduced to the structure through by

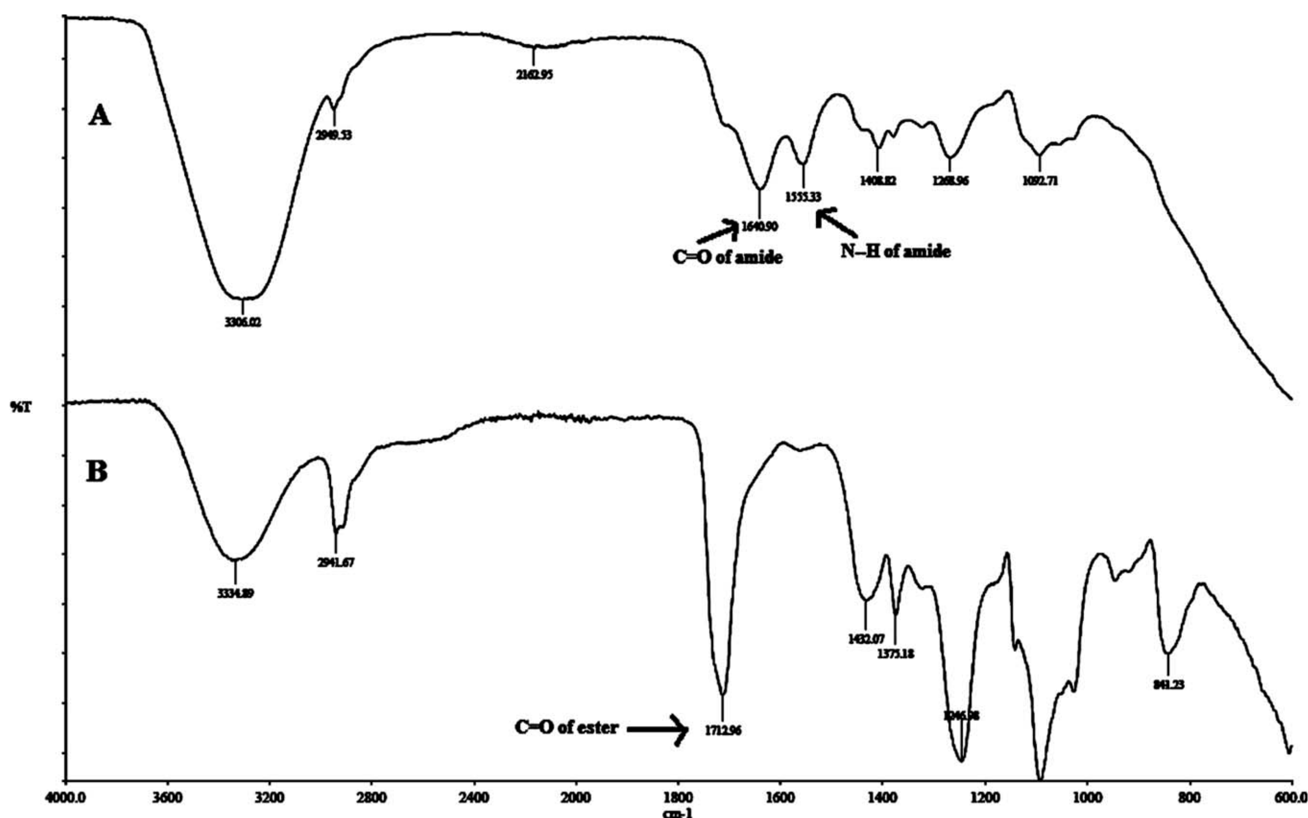

Figure 2. FT-IR spectra of (A) enzyme -immobilized nanofibers and (B) thermally crosslinked PVA-PAA nanofiber

covalent immobilization of the enzyme. Therefore, the presence of the nitrogen was mainly attributed to enzyme.

Immobilization Efficiency

There are several studies including α-amylase immobilization in which the binding capacity of the support materials is labile due to the characteristic properties of the prepared materials. In our previous studies, on α-amylase immobilization capacities onto amine functionalized glass beads,[16] UV curable N-(4-sulfophenyl)-maleimide polymeric films,[17] glutaraldehyde activated silanized calcium carbonate were reported[18] as 25.2, 68.18, and 199.43 mg/g support material, respectively. In this study, the amount of the covalently bonded enzyme was found to be as 181.48 ± 3.60 mg/g of hybrid nanofiber.

Effect of pH on Activity

The activity of an enzyme may be modified by its immediate microenvironment. An enzyme in solution can have a different optimal pH from the same enzyme immobilized on a solid matrix depending on the surface and residual charges on the solid matrix and the nature of the enzyme-bound pH value in the immediate vicinity of the enzyme environment. A change in the optimum pH normally results in insolubilization of enzyme, depending upon the polymer used as support.[16]

The effect of pH on the free and immobilized α-amylase was investigated in the range of pH 5.0–8.0, and results were presented in Figure 4. The maximum activity was observed at pH 6.5 for free α-amylase. However, the maximum pH of the immobilized enzyme was shifted 0.5 pH unit to the acidic region. The shift depends on the enzyme reaction as well as on the structure and the charge of the matrix. For amylase immobilization, shift toward the acidic or basic directions have been previously observed.[19] In our previous work, the pH shift of the bound enzyme was found to be 1 pH unit to the acidic region.[16]

Effect of Temperature on Activity

Temperature is one of the major factors which affect biological activity of proteins. Inactivation of enzyme by temperature variation is due to conformational changes in the protein molecular structure.[20] The activity of the free and immobilized α-amylase was assayed at various temperatures (30–80°C). As it can be seen in Figure 5, it was found that the optimum temperature for the immobilized α-amylase was ∼ 50°C, which was 20°C higher than that of the free one. The free and the immobilized enzyme exhibited different temperature profile. The results were showed that the immobilized enzyme had 77.9% activity at 60°C, while the free enzyme had only 62.03%. However, there was a sharp difference in activity loss between free and immobilized enzyme, which was ∼ 24.4% at 70°C. Finally at 80°C, the relative activity was calculated as ∼ 12.4% for immobilized enzyme and 4.8% for the free one. The immobilized enzyme had a higher activity at high temperatures (50–80°C) than the free one. Thus, immobilized enzyme was much more stable than the free enzyme at higher temperatures. We have found similar results in our previous studies according to the thermal stability of immobilized α-amylase in which optimum temperature is ∼ 50°C.[16,17]

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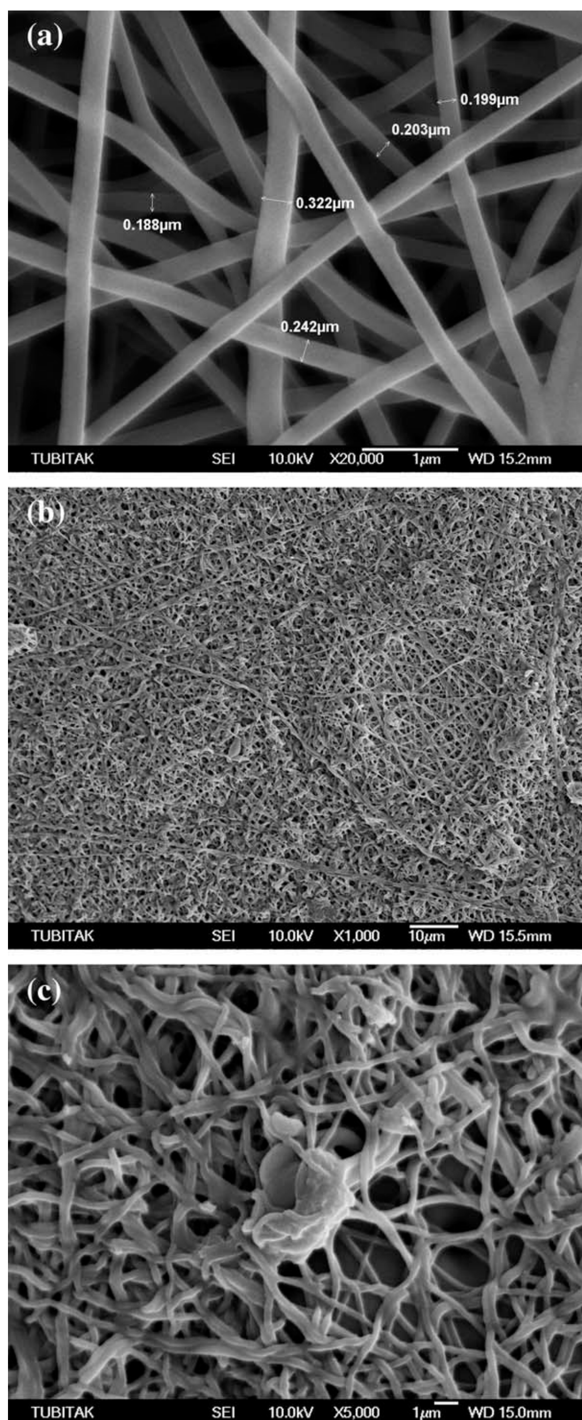

Figure 3. (a) SEM micrographs of thermally crosslinked PVA-PAA nanofiber average diameter; (b) and (c) SEM micrographs of the enzyme immobilized PVA-PAA nanofiber.

Increased thermal stability has been reported for a number of immobilized enzymes, and the support material is supposed to preserve the tertiary structure of the enzyme. The authors demonstrated that the thermal stability of enzymes might be drastically increased if they are attached to a complementary surface of a relatively rigid support in a multipoint.[21] Enhanced thermal stability is due to the reduced mobility of the covalently immobilized enzyme as it is protected from conformational changes caused by the environment.[22,23]

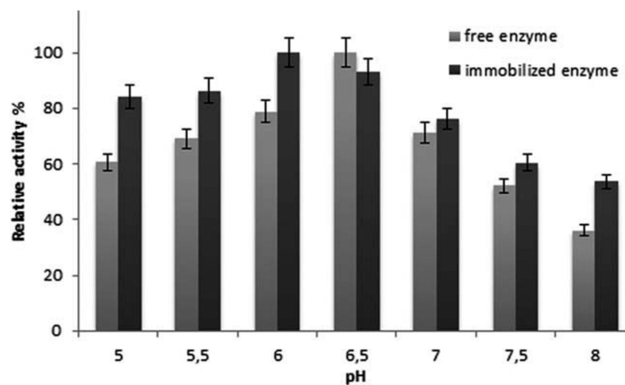

Figure 4. Effect of pH on activity free and immobilized α-amylase.

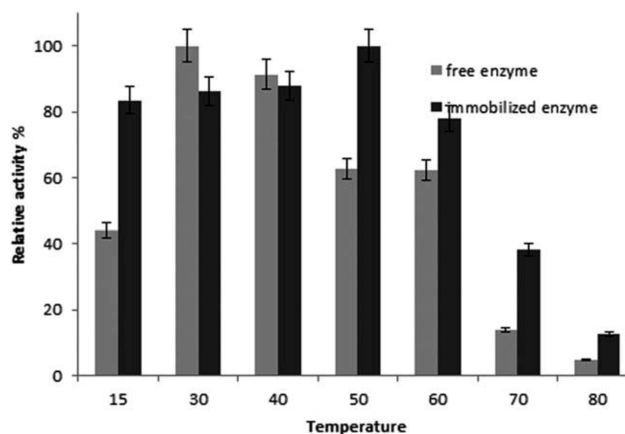

Figure 5. Relative enzyme activity as a function of temperature for free and immobilized α-amylase.

Kinetics

Mechanisms that influence the interactions between biological substrates and enzymes are essential to engineer biomolecular processes.[24] The kinetic parameters of the free and immobilized amylases, V_{max} and K_m values, were assayed using various initial substrate concentrations from 0.25 to 2.5 mg/mL. K_m values were found as 0.55 and 0.86 mg/mL for free and immobilized

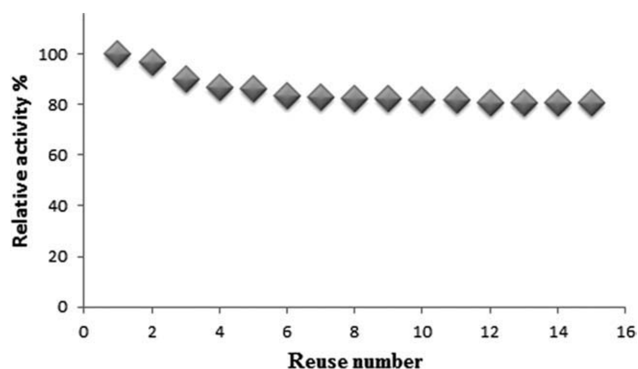

Figure 6. Reusability of immobilized α-amylase.

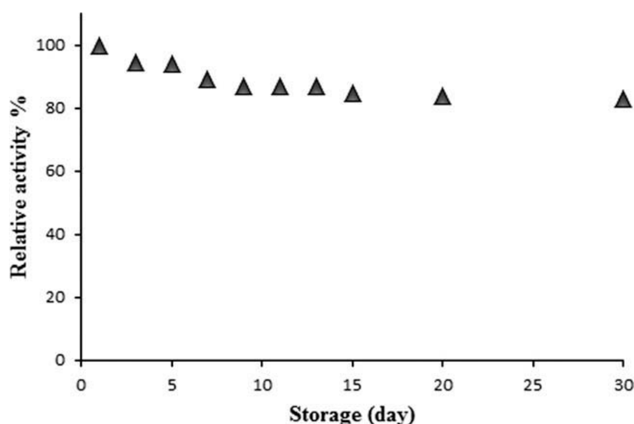

Figure 7. Effect of storage time on activity immobilized α-amylase.

enzymes, respectively. The K_m value is known as the affinity of the enzymes to substrates and the lower values of K_m emphasize the higher affinity between enzymes and substrates.[25,26] The results have shown that the affinity of the α-amylase to its substrate was decreased by immobilization. The large K_m value after enzyme immobilization, caused by the conformational changes of the enzyme, resulted in a lower affinity for substrate contact. This also may be caused by the lower accessibility of the substrate to the active site of the immobilized enzyme by the increased diffusion limitations.[27] V_{max} values for the free and immobilized enzymes were calculated as 10 and 0.202 mg/mL min^{-1}, respectively.

Reusability and Storage Stability

Enzymes are quite sensitive against the environmental situation and may lose their activities easily when compared with the immobilized enzymes. Therefore, it is significant to characterize their reusability and storage stability for preparative or industrial uses. The capability of the immobilized enzyme to remain active was examined by recording the changes in activity after repeated washes. Activity found for each repetition was compared with the initial activity, assuming it was 100%, and results were given in Figure 6. The residual activity of the immobilized α-amylase was decreased with the increasing number of washes. At the end of 10th cycle, the residual activity retained was ~ 81.7%. The immobilized enzyme had excellent operational stability, the activity of which remained above 80% of the initial state after 15th use. In our previous study, it was found that the reuse capabilities of α-amylase were in the range of 78–85% when immobilized on UV curable N-(4-sulfophenyl)-maleimide polymeric films[17] and %75 when immobilized on functionalized glass beads.[16]

Storage stability is one of the significant indexes to evaluate the properties of enzyme. Generally, enzymes are not stable in solution and their activities could decrease during storage. It has been shown that the storage stability of the enzyme increases on immobilization with polymeric supports.[28,29]

In Figure 7, the storage stabilities of immobilized amylase can be seen. The free and immobilized enzymes were stored in 10 mM phosphate buffer (pH 6.0) at 4°C, and their activities were tested for 30 days. Free enzyme lost its activity completely within 15 days. However, the immobilized enzyme retained 82.9% of its activity after 30 days. These results showed that by immobilization, the enzyme gained more stable character than the free one. Retained activities of α-amylase immobilized on various polymeric supports were previously reported as 75–90% after 25 days of storage.[30,31] Thus, the support and technique of immobilization provided a longer shelf life than that of free counterpart.[32]

CONCLUSION

α-amylase covalently immobilized onto the thermally crosslinked electrospun PVA/PAA nanofibers. Thermally crosslinked electrospun PVA-PAA nanofiber became insoluble in water and it has a good chemical stability and mechanical strength. SEM micrograph, SEM-EDS, FTIR spectra, and Bradford protein assay confirmed that the enzyme was covalently bonded on nanofiber surface. The covalent bonding between nanofiber surface and enzyme changes fiber morphology. The immobilized enzyme exhibited better thermostability than the free one. The optimum pH of the immobilized enzyme was shifted 0.5 pH unit to the acidic region. The storage stability and reusability improved by the immobilization on this enzyme support. These results confirm that α-amylase was successfully immobilized and gained more stable character compared to free one. The results demonstrated the surface functionalized electrospun nanofibrous material could be used as a promising material for immobilizing a wide range of bioactive molecules.

REFERENCES

1. Gupta, R.; Gigras, P.; Mohapatra, H.; Goswami V. K.; Chauhan B. *Process Biochem.* **2003,** *38*, 1599.
2. Reshmi, R.; Sanjay, G.; Sugunan, S. *Catal. Commun.* **2006,** *7*, 460.
3. Aksoy, S.; Tumturk, H.; Hasirci, N. J. *Biotechnol.* **1998,** *60*, 37.
4. Lim, L. H.; Macdonald, D. G.; Hill, G. A. *Biochem. Eng. J.* **2003,** *13*, 53.
5. Marinov, I.; Gabrovska, K.; Velichkova, J.; Godjevargova, T. *Int. J. Biol. Macromol.* **2009,** *44*, 338.
6. Wan, L. S.; Ke, B. B.; Xu, Z. K. *Enzym. Microb. Tech.* **2008,** *42*, 332.
7. Wan, L. S.; Ke, B. B.; Wu, J.; Xu, Z. K. *J. Phys. Chem. C* **2007,** *111*, 14091.
8. Chen, P. J.; Ho, H. K.; Chiang, P. Y.; Kuo, W. W. *J. Membr. Sci.* **2009,** *340*, 9.
9. Koski, A.; Yim, K.; Shivkumar, S. *Mater. Lett.* **2004,** *58*, 493.
10. Kim, D. S.; Park, H. B.; Rhim, J. W.; Lee, Y. M. *Solid State Ionics* **2005,** *176*, 117.
11. Li, L.; Hsieh, Y. L. *Nanotechnology* **2005,** *16*, 2852.
12. Akdemir, Z. S.; Demir, S.; Kahraman, M. V.; Apohan, K. N. *J. Mol. Catal. B: Enzym.* **2011,** *68*, 104.
13. Bradford, M. M. *Anal. Biochem.* **1976,** *72*, 248.

14. Bernfield, P. In Advances in Enzymology; Nord, F.Ed.; Interscience Publication: New York, **1951**; Vol. *12*, 379.
15. Kim, T. G.; Park, T. G. *Biotechnol. Progr.* **2006,** *22*, 1108.
16. Kahraman, M. V.; Bayramoglu, G.; Kayaman-Apohan, N.; Gungor, A. *Food Chem* **2007,** *104*, 1385.
17. Yarar, Ü.; Kahraman, M. V. *J. Appl. Polym. Sci.* **2009,** *114*, 3716.
18. Demir, S.; Gök, B. S.; Kahraman, M. V., *Starch/Starke* **2012,** *64*, 3.
19. Bayramoğlu, G.; Yılmaz, M.; Arica, M. Y. *Food Chem.* **2004,** *84*, 591.
20. Tanford, C. *Adv. Prot. Chem. Str.* **1968,** *23*, 121.
21. Martinek, K.; Kilbanov, A. M.; Goldmacher, V. S.; Berezin, I. V. *Biochim. Biophys. Acta* **1977,** *485*, 1.
22. Emi, S.; Murase, Y.; Hayashi, T.; Nakajima, A. *J. Appl. Polym. Sci.* **1990,** *41*, 2753.
23. Wang, F.; Guoa, C.; Liu, H. Z.; Liu, C. Z. *J. Mol. Catal. B: Enzym.* **2007,** *48*, 1.
24. Swartz, H. M.; Bolton, J. R.; Borg, D. C. Biological Applications of Electron Spin Resonance; John Wiley & Sons Inc.: NJ, U.S.A., **1972;** 251.
25. Cosulich, M. E.; Russo, S.; Pasquale, S.; Mariani, A. *Polymer* **2000,** *41*, 4951.
26. Park, D.; Haam, S.; Jang, K.; Ahn, I. S.; Kim, W. S. *Process Biochem.* **2005,** *40*, 53.
27. Li, S. F.; Chen, J. P.; Wu, W. T. *J. Mol. Catal. B: Enzym.* **2007,***47*, 117.
28. Cetinus, S. A.; Sahin, E.; Saraydin, D. *Food Chem.* **2009,** *114*, 962.
29. Tuncagil, S.; Kayahan, S. K.; Bayramoglu, G.; Arica, M. Y.; Toppare, L. *J. Mol. Catal. B: Enzym.* **2009,***58*, 187.
30. Kahraman, M. V.; Kayaman-Apohan, N.; Ogan, A.; Güngör, A. *J. Appl. Polym. Sci.* **2006,** *100*, 4757.
31. Tümtürk, H.; Aksoy, S.; Hasırcı, N. *Food Chem.* **2000,** *68*, 259.
32. Abrol, K.; Qazi, G. N.; Ghos, A. K. *J. Biotechnol.* **2007,** *128*, 838.