Soybean Oil Based Resin: A New Tool for Improved Immobilization of α-Amylase

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ABSTRACT: Acrylated epoxidized soybean resin has been utilized to immobilize the α-amylase via UV-curing technique. Among the numerous methods that exist for enzyme immobilization, entrapment and covalent binding are the focus of this study. The properties of immobilized enzyme were investigated and compared with those of the free enzyme. Upon immobilization by the two methods, the catalytic properties of the enzyme were not considerably changed as compared with that of nonimmobilized form; only the pH profile was broadened for the immobilized enzyme. The free enzyme lost its activity completely in 20 days, where as storage and repeated usage capability experiments demonstrated higher stability for the immobilized form. Immobilized enzyme prepared by attachment method possesses relatively higher activity compared with the activity of those obtained by entrapment method. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 100: 4757–4761, 2006

Key words: epoxy acrylates; UV-curing coating; α-amylase; immobilization; enzyme activity

INTRODUCTION

Enzyme engineering is a fast-growing application in the pharmaceutical and food markets. Enzymes can be operated in the liquid form or immobilized on various supports. The main advantages of immobilized enzymes are reusability, rapid termination of reactions, controlled product formation, easy separation from the reaction mixture, minimized enzyme loss, and greater efficiency in consecutive multistep reactions.[1–3]

The methods of immobilization can be classified into four main categories: matrix entrapment, microencapsulation, adsorption, and covalent binding. Many entrapment methods are used today, and all are based on the physical occlusion of enzyme molecules within a network structure such that the diffusion of enzyme molecules to the surrounding medium is severely limited. Unlike the adsorption and covalent-binding methods, entrapment method does not directly involve the formation of bonds between the support material and enzyme molecules.[4] This results in a wide applicability. On the other hand, covalent attachment is also an important method that has been demonstrated to induce higher resistance to temperature, denaturants, and organic solvents in several cases.[5] However, it may alter the conformational structure and activity center of the enzyme, resulting in major loss of activity.[6]

Epoxy supports have been widely used as carriers for immobilized enzymes. Their advantages are accessibility, cheapness, and high stability during storage. They are also able to form very strong linkages with enzymes and proteins.[7–9] Recently, it was reported that UV-curing technique used for enzyme entrapment provides very convenient method and causes less enzymatic-activity loss.[10] In this article, the attention is focused on immobilization of α-amylase on a UV curable bio-based resin via both entrapment and covalent-binding methods. Soybean oil is one of the many readily available renewable resources.[11] Economical and environmental advantages of this material over petroleum-based ones was making it an attractive alternative. For this reason, an acrylated epoxidized soybean oil (AESO) was synthesized as an enzyme support and characterized. The effects of the immobilization method on enzyme activity, optimum pH, and storage stabilities of the immobilized α-amylase were investigated.

EXPERIMENTAL

Materials

Epoxidized soybean oil (epoxy content 4.037 mol/kg) was kindly supplied by İldeş Kimya (Kocaeli, Turkey). Acrylic acid was obtained from Henkel (Turkey). Triphenyl phosphine (TPP) was purchased from Fluka

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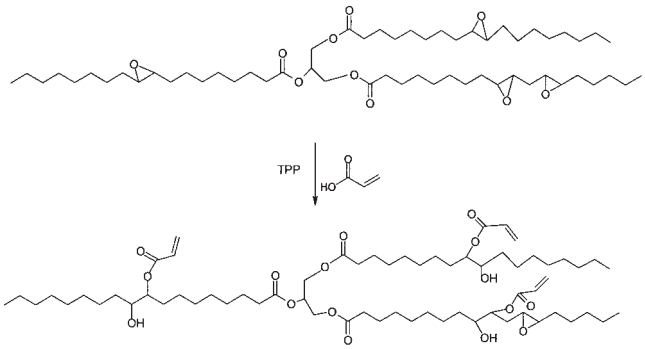

Scheme 1 Synthesis of AESO.

AG. Hydroquinone was received from Merck AG. α-Amylase (1,4-α-D-glucan-glucanohydrolase; EC 3.2.1.1, Type VI-B from porcine pancreas, extra pure 35 U/mg), starch, maltose, and 3,5-dinitrosalicylic acid (DNSA) were all obtained from Sigma Chemical. 1-Hydroxy cyclohexyl phenyl ketone (Irgacure 184) was supplied by Ciba Specialty Chemicals (Turkey). All other chemicals were of analytical grade and were purchased from Merck AG. Freshly double-distilled water was used throughout.

Synthesis of acrylated epoxidized soybean oil

Epoxidized soybean oil (200 g), TPP (2.52 g), and hydroquinone (100 mg) were charged into a three-necked 500-mL round-bottom flask equipped with a nitrogen inlet and dropping funnel. Acrylic acid (52.5 mL, 1/0.95 equivalent to total epoxy content) was then added dropwise to the well-stirred reaction mixture over a period of 1 h. The reaction mixture was kept at 80°C and allowed to react for additional 5 h. At the end of the reaction, the resulting resin was analyzed by FTIR and ^{1}H NMR. The characteristic —C═C— double band absorption at 1615–1630 cm^{-1} confirmed that the reaction had occurred successfully, and also disappearance of —COOH protons at 10 ppm demonstrates that all acrylic acid had reacted with epoxides. Epoxy content of resin was found as 1.65 mol/kg. The resin was stored at 4°C until use. A representation of this reaction is shown in Scheme 1.

FTIR (liquid, cm^{-1}): 3467 (secondary, —OH), 2929–2848 (aliphatic —CH—), 1733 (—C═O), 1615–1630 (acrylic —C═C—).

^{1}H NMR ($CDCl_3$, ppm): δ 0.9 (—CH_3), 1.1–1.6 (aliphatic —CH_2—), 2.3 (fatty acid protons α to the carbonyl groups), 4.2–4.5 (—O—CH_2— in triglyceride), 4.9 (—CH—O— in triglyceride), 5.8–6.6 (acrylate protons).

Polymeric film preparation: Immobilization of α-amylase by UV-curable resin

The UV-curable acrylated epoxidized soybean oil and α-amylase powder were mixed in the ratio of 9/1 wt/wt. Thereafter, free radical initiator, Irgacure-184, was added in the amount of 2.0 wt % of the total mixture weight. Polymeric film was obtained by pouring the viscous liquid mixture onto Teflon®-coated mold after 0, 4, and 24 h coupling duration time. To prevent the inhibiting effect of oxygen, mixture in the mold was covered by transparent, 25-μm thick Teflon™ film. Before irradiation with a high-pressure UV-lamp (Osram Ultra-Vitalux, 300 W, 230 V AC E27 FS1), a quartz glass plate was placed over the Teflon film to obtain a smooth surface with desired thickness. The nominal wavelength of the lamp used was 350 nm. Finally, the formulation was irradiated for 200 s under UV lamp. Immobilized enzymes were stored at 4°C until use.

Assay of α-amylase activity

α-Amylase activity was measured based on the assay suggested by Bernfield.[12] Briefly, 1 wt % starch solution was prepared by dissolving soluble starch in 100 mL of 0.02*M* sodium phosphate buffer (pH = 6.9) with 0.006*M* NaCl. A small piece of polymeric film (~1 cm^2) containing amylase enzyme was placed into a test vial. Then 0.5 mL of starch solution was added, and the system was incubated in a water bath with constant shaking at 25°C for exactly 5 min. After that the polymeric film was taken outside from the vial. The reaction was stopped by adding 1 mL of 3,5-DNSA reagent. Incubation was performed in a boiling water-bath for 5 min. After dilution with distilled water, 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 (U) was defined as the amount of enzyme liberating 1 mg maltose/min under the assay conditions. The immobilized enzyme had to be washed with distilled water before remeasuring its activity in successive used cycles. To determine the pH profiles for the free and immobilized α-amylase activity, assays were carried out over the pH range of 5–8. The results of dependence of pH, storage, and used-up capacity are presented in a normalized form, with the highest value of each set being assigned the value of 100% activity.

RESULTS AND DISCUSSION

In this work a new enzyme carrier was prepared by photoinitiated polymerization of AESO via UV-curing technique. AESO was synthesized as a starting material to increase the shelf life of immobilized enzyme. TPP was used as catalyst. Scheme 1 illustrates the general synthetic procedure used for AESO. After the acrylation reaction, the triglyceride contains both residual amounts of unreacted epoxy rings as well as

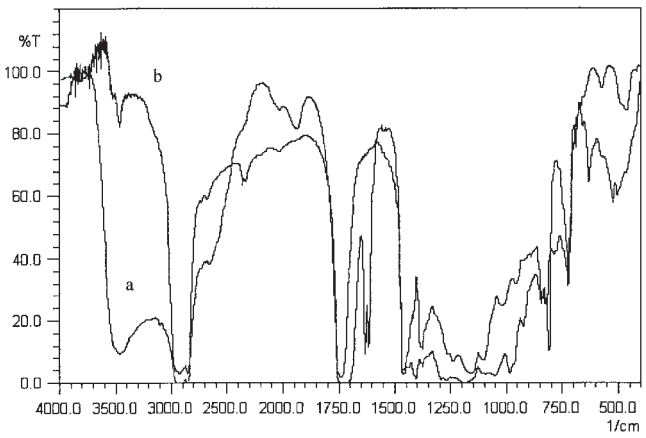

Figure 1 FTIR spectrum of (a) AESO and (b) epoxidized soybean oil.

newly formed hydroxyl groups. The chemical structure of resulting resin was characterized by FTIR and ^{1}H NMR spectroscopy techniques. As can be seen in Figure 1, the appearance of the absorption band at 3467 cm^{-1}, which is assigned to the secondary hydroxyl group peak, indicates the opening of oxirane ring. In addition, characteristic —C═C— absorption bands appeared at 1615–1630 cm^{-1}.

The ^{1}H NMR spectrum of AESO is shown in Figure 2. One can see that the spectrum presents all the hydrogen signals expected. The peaks at 5.8–6.6 ppm represent the three protons attached to the —C═C— bond of the acrylate esters. The peaks between 4.2 and 4.5 ppm represent the two glycerol methylene groups, and the weak multiplet centered at about 4.9 ppm arises from the resonance of the glycerol methine peak. The negligible amount of epoxy rings left after acrylation would produce multiplets in the chemical shift range from 2.6 to 3.2 ppm. Hydroxyl groups attached to methine protons were seen as multiplets at 5.5 ppm. The methylene protons adjacent to carbonyl group of the fatty acid and terminal methyl groups resonate as strong multiplets at 2.3 and 0.9 ppm, respectively.

The entrapment of α-amylase in polymeric matrix was achieved via UV curing of acrylated resin in the presence of an initiator. The water absorption value was found as 0.98 wt/wt %. Polymeric matrix prepared from AESO resin is assumed to be a very good candidate as a rigid support because of its low water absorption value and negligible shrinkage behavior. It has been already reported that the usage of rigid support for enzyme fixation preserved the activity of the fixed biomolecule as compared with the usage of flexible ones.[13]

Figure 3(a) and (b) shows the SEM images of the enzyme support at two different magnifications. It can be seen that the fractured surface of the epoxy support

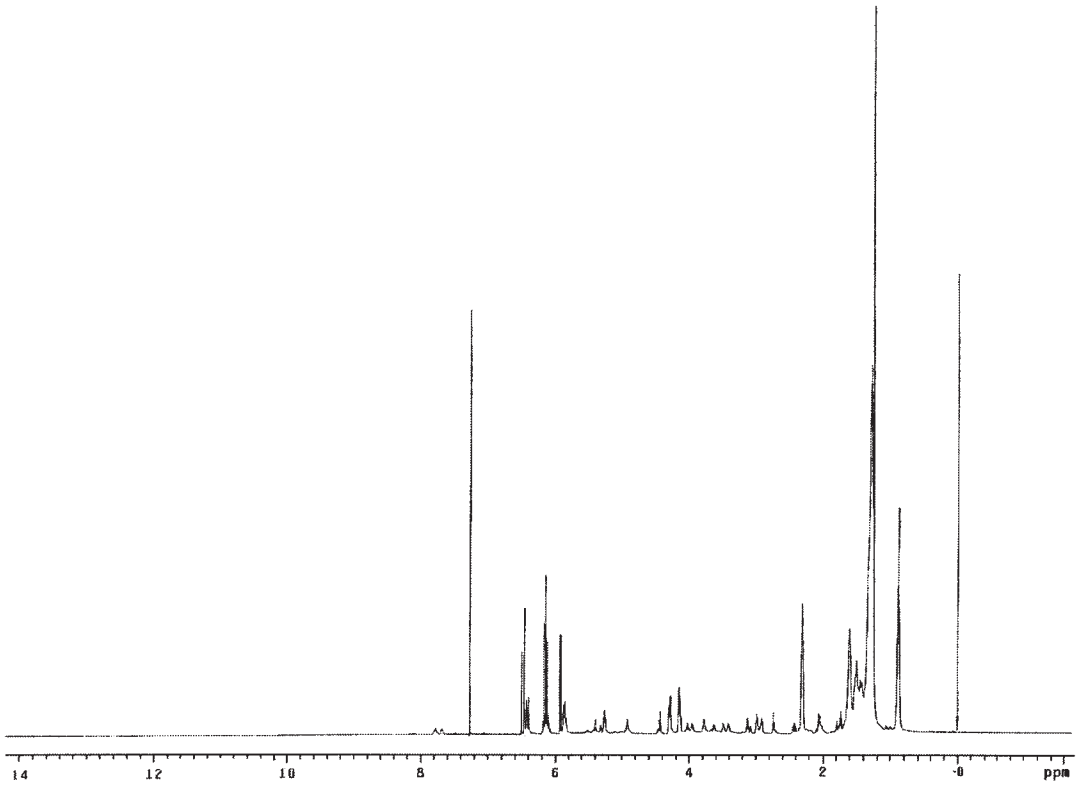

Figure 2 ^{1}H NMR spectrum of AESO.

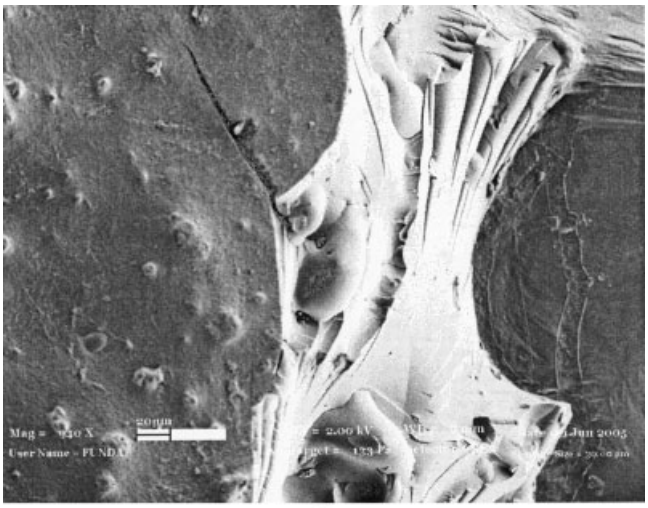

Figure 3 SEM micrographs of the enzyme support with magnifications (a) ×940 and (b) ×9480.

presents uniform crack direction and reveals the characteristic of brittle failure. Figure 3(a) also shows a rough surface containing dispersed phase particles probably due to the amylase powder. At higher magnification, it is observed that these crater-like particles are homogeneously distributed through the surface and have a very small size, less than 10 μm in diameter.

The effect of pH on the activity of free and immobilized α-amylase for starch hydrolysis was determined in the pH range between 4.0 and 8.0 at 25°C. The results are presented in Figure 4. The maximum activity for free enzyme was observed at pH = 6.5. After immobilization, the optimum pH did not change. However, the broad pH profile means that the immobilized enzyme has higher relative activity than that of its native analogue.[4,14] This result may be attributed to the stabilization of α-amylase molecules, resulting from conformational restrictions inside the polymeric matrix.

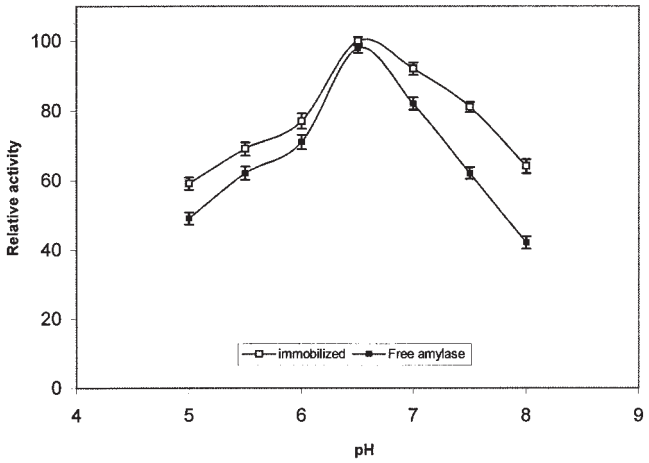

Figure 4 The effect of pH on the activity of the immobilized and free α-amylase.

The effect of storage conditions on the activity of the immobilized enzyme is an important aspect to ensure that a long shelf-life is possible. The decrease of enzyme activity was essentially identical for all storage condition tested.[5] In Figure 5, the storage stabilities of free and immobilized α-amylase at pH = 6.9 can be seen. The free and the immobilized α-amylase were stored at 4°C, and the activity measurement was carried out for a period of 25 days. The free enzyme lost all its activity within 20 days. The retained activity of immobilized enzyme was found to be around 80% after 25 days storage period. Immobilization definitely put the enzyme into a more stable position in comparison with free enzyme. The enhanced storage condition of the enzyme can be attributed to higher conformational stability of enzyme due to entrapment within the crosslinked network. It should also be noted that the immobilized α-amylase preserved about 95% of its

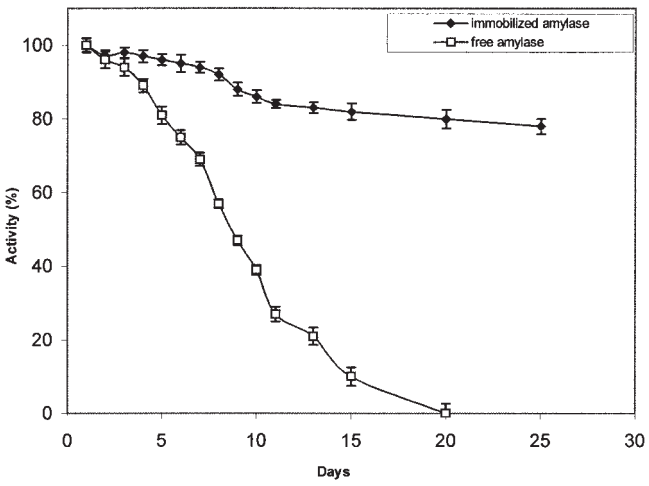

Figure 5 The effect of storage on the activity of immobilized α-amylase (pH = 6.9).

initial activity during 2 months storage period at room temperature. Thus, it can be concluded that entrapment method provides extended shelf-life with respect to the free counterpart.

The effects of coupling time on the immobilized enzyme stability can be seen in Figure 6. The immobilized enzyme activity yield was markedly higher at longer coupling times. It was deduced that the amylase enzyme could be attached to AESO resin through ring opening of residual epoxy groups. Covalent attachment of enzyme under very mild experimental condition permits a very intense enzyme-support interaction. Therefore, by using attachment method higher enzyme stability is achieved compared with that obtained using enzyme entrapment.

The most important advantage of immobilization is repeated use of enzymes. The immobilized samples that were prepared by entrapment and attachment methods were used repeatedly 50 times within 8 h and the measured activities are shown in Figure 7 (pH = 6.9). It was observed that after the fifty uses, immobilized samples retained 74 and 88% of their initial activities. From these results, it was seen that covalent binding method definitely provides better enzyme stability on the reuse experiments.

CONCLUSIONS

The present work introduces a new enzyme-carrier based on a UV-curable AESO. α-Amylase has been successfully immobilized onto the carrier by both entrapment and covalent-binding methods. Immobilization is much more rapid by using UV-curable support material. Retained enzyme activity has been found to be much higher using the new support than in preparations using the conventional ones in many cases. Furthermore, storage stability and reuse numbers significantly improved by the immobilization on this support, compared with those of the free enzyme. Therefore, this support can be recommended as a new tool for enzyme immobilization.

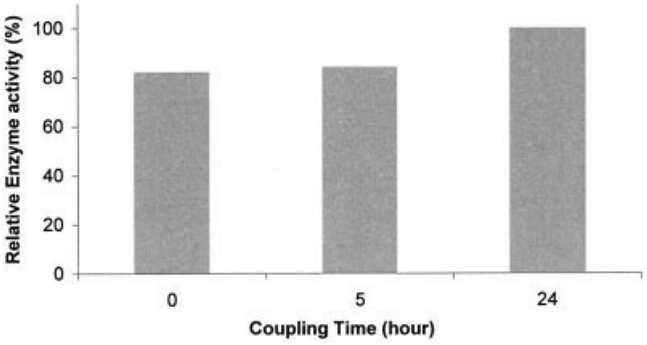

Figure 6 The effect of the coupling time on enzyme stability.

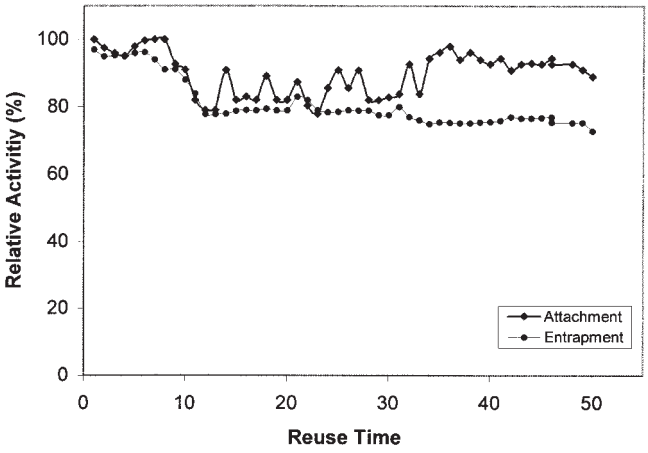

Figure 7 Reuse of immobilized α-amylase (pH = 6.9).

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