Covalent Immobilization of α-Amylase onto UV-Curable Coating

Ümran Yarar, Memet Vezir Kahraman

Department of Chemistry, Faculty of Arts and Science, Marmara University, Istanbul 34722, Turkey

Received 2 August 2008; accepted 19 March 2009
DOI 10.1002/app.30442
Published online 17 August 2009 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: A UV-curable *N*-(4-sodiumsulfophenyl)-maleimide monomer was synthesized, and its potential for enzyme binding was investigated. The bromine, which is used to activate the synthesized monomer for covalent attachments, has the advantage of giving reaction with the surface groups of enzyme under very mild conditions (0°C, 30 min). In this procedure, sulfonyl bromide pendant monomer reacted with amino groups of the protein to form sulfonamide bonds. Polymeric support was prepaped by UV-curing technique. The water adsorption value was found to be less than 1%. The enzyme-bounding yield was found to be 68.18 ± 4.20 mg/g monomer. The maximum activity was observed at pH 6.5. Immobilization did not change the pH-dependency of the enzyme activity. It was found that the optimum temperature for the free enzyme was ~ 30°C, whereas it shifted to nearly 50°C for the immobilized enzyme. Free enzyme lost its activity completely within 15 days. Immobilized enzyme lost only 30% of its activity in 30 days. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 114: 3716–3722, 2009

Key words: α-amylase; covalent immobilization; UV curing coating; sulfon amide

INTRODUCTION

Enzymes are relatively expensive reaction components, and therefore they increase the production costs. They are also extremely sensitive to environment conditions and can be easily denatured. In spite of their excellent catalytic properties, enzyme properties usually have to be improved before their implementation at industrial scale. In industrial reactors, generally, soluble enzymes have to be immobilized for reusing. Other critical enzyme properties, like stability, activity and inhibition by reaction products, and selectivity toward nonnatural substances, have to be improved.[1] Immobilization refers to the preparation of insoluble biocatalytic derivatives and involves the coupling of enzymes to solid supports that are either organic or inorganic. There are many advantages of the immobilization of proteins onto solid support[2–4]:

- Improve enzyme shelf-life (half-life);
- Improve stability in adverse reaction conditions;
- Improve stability in the presence of organic solvents;
- Easy separation from product stream;
- Allow continuous flow operations and repetitive usage;
- Increase enzymatic activity, in few cases.

The methods of immobilization can be classified into four main categories: matrix entrapment, micro encapsulation, adsorption, and covalent binding. More efficient immobilization methods involve the covalent attachment of the enzyme onto or into a solid support.[5]

Covalent immobilization is accomplished through the binding of reactive groups on the enzyme with a chemically active surface. Covalent binding of enzymes on solid supports has some advantages, such as increase of thermal and storage stability, prevention of the leakage, and decrease of diffusion problems for the substrates and products in the reaction medium. Immobilization is often accompanied by some changes in the optimum conditions for optimum enzymatic activity, such as temperature, pH, and substrate concentration. The extent of these changes depends on the type of enzymes, support materials, and activators, as well as the type and conditions of immobilization process.[6] A nonconventional strategy to achieve multipoint covalent immobilization within a polymer network is supplied by copolymerizing the enzyme with monomers capable of a chemical reaction with specific functionalities on its surface. During polymerization, the enzyme acts as a part of the monomer and is thus expected to be distributed within the resulting biopolymer.[5] There

Correspondence to: M. V. Kahraman (mvezir@marmara.edu.tr).

Contract grant sponsor: Marmara University, Commission of Scientific Research Project (M.Ü.BAPKO); contract grant number: FEN-YLS-120707-0155.

© 2009 Wiley Periodicals, Inc.

have been many reports about covalent immobilization of α-amylase used for the hydrolysis of starch.[7–11]

In this study, α-amylase was immobilized onto a new support material, which could be a good candidate for rigid support systems. For this purpose, UV-curable *N*-(4-bromosulfophenyl)-maleimide monomer was synthesized to bind enzyme. This monomer provides reactive sulfonyl bromide functionalities for covalent biomolecule immobilization under very mild conditions (0°C, 30 min). Afterwards, polymeric support was prepaped by UV-curing technique. The enzyme activity of free and immobilized α-amylase was examined at different pHs (5.5–8.0) and temperatures (30–90°C). The storage stability and reusability of the covalently immobilized enzyme was studied and analyzed. Each assay reported in this article is an average of three separate experiments.

EXPERIMENTAL

Materials

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, 3,5-dinitrosalicylic acid (DNSA), and bovine serum albumin (BSA) were all obtained from Sigma Chemical (Labor Teknik A.S., Istanbul, Turkey). Maleic anhydride and vinyl-2-pyrolidone (NVP) were purchased from Fluka (Labor Teknik A.S., Istanbul, Turkey). Polyethyleneglycol-diacrylate (MW = 258 g/mol) and polyethyleneglycol (PEG)-monoacrylate (MW = 375 g/mol) were purchased from Aldrich (Labor Teknik A.S., Istanbul, Turkey). Coomassie® Brilliant Blue G-250 was obtained from Bio-Rad. 1-Hydroxy-cyclohexyl-phenyl-ketone (Irgacure 184) was supplied by Ciba Specialty Chemicals (Istanbul, Turkey). All other chemicals were of analytical grade and were purchased from Merck AG (Labor Teknik A.S., Istanbul, Turkey). Freshly double distilled water was used throughout.

Characterization

The chemical structures were identified by Fourier transformed infrared (FTIR) and nuclear magnetic resonance (NMR) spectroscopy. ^{13}C-NMR spectrum was obtained by using Varian model T-60 NMR spectrometer operated at 200 MHz. FTIR spectrum was recorded on Shimadzu 8300 FTIR spectrometer. Scanning electron microscope (SEM) imaging was performed on a JEOL JSM-5919 LV. Elemental concentrations for carbon, nitrogen, and oxygen were evaluated with an energy dispersion spectrum (EDS; model no. 7274; Oxford Instruments-Inca). UV–visible spectrum was performed on Shimadzu 1601 spectrophotometry.

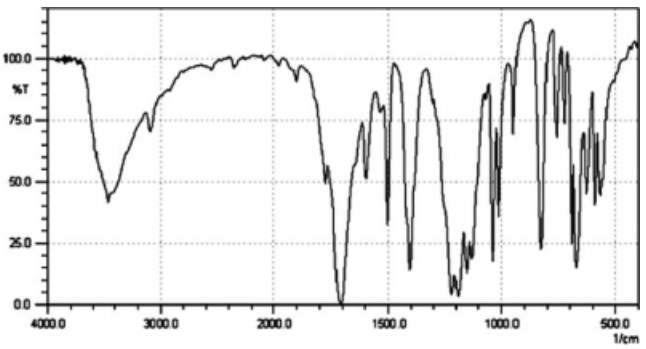


Figure 1 FTIR spectrum of *N*-(4-sodiumsulfophenyl)-maleimide monomer.

Synthesis of the *N*-(4-sodiumsulfophenyl)-maleimide

The *N*-(4-sodiumsulfophenyl)-maleimide monomer is prepared by a two-step process. In the first step, 10.32 g maleic anhydride was reacted with 18.74 g sulfanilic acid sodium salt in a dry methanol (200 mL) at room temperature (1 h) to form a half imide under nitrogen atmosphere.[12] The formed precipitate of the half imide was recovered by filtration and dried under high vacuum at 40°C.

In the second step, 5 g (23.47 mmol) half imide, 0.43 g sodium acetate, and 85 mL acetic anhydride were added into a three-necked, 250-mL, round-bottom flask, equipped with a nitrogen inlet. The mixture was stirred at 85°C for 6 h. After cooling, the *N*-(4-sodiumsulfophenyl)-maleimide is obtained as a precipitate. The product was filtered and washed with 52 mL ethanol and 35 mL acetone and dried under high vacuum at 40°C. FTIR and ^{13}C-NMR spectra of synthesized *N*-(4-sodiumsulfophenyl)-maleimide were given in Figures 1 and 2, respectively. A representation of this reaction is shown in Scheme 1.

Coupling of α-amylase onto maleimid monomer

Br_2 (0.52 mL, 10 mmol) was carefully added dropwise into a suspension of 2.62 g Ph_3P (10 mmol) in 10 mL MeCN at 0°C under nitrogen atmosphere. Then 2.75 g *N*-(4-sodium sulfophenyl)-maleimide (10 mmol) was added in portions to the mixture at room temperature. Afterwards, functionalized monomer was reacted with α-amylase enzyme (190 mg) for 30 min at 0°C. Then, 40 mL water was added to the mixture and extraction performed with 3 × 50 mL dichloromethane. The organic phase containing enzyme-bounded maleimide monomer was dried over Na_2SO_4, and solvent was evaporated under reduced pressure at room temperature.[13] A representation of this reaction is shown in Scheme 2. The washing solution (water phase) was kept for measuring the amount of immobilized enzyme. The

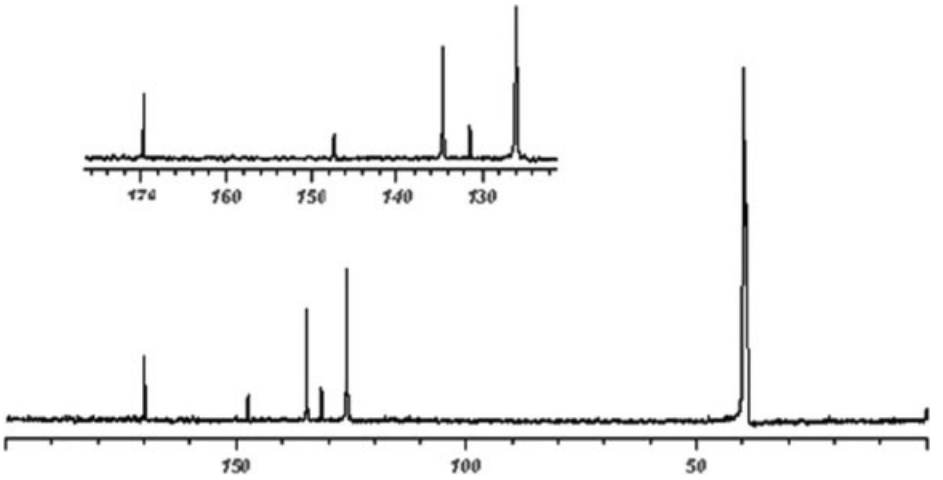


Figure 2 ^{13}C-NMR spectrum of *N*-(4-sodiumsulfophenyl)-maleimide monomer.

enzyme protein concentration within the extract was measured by using Coomassie Brillant Blue reagent as described by Bradford.[14] A calibration curve was prepared with BSA solution. The bounded α-amylase was calculated from the difference between the amount of enzyme in the initial feed and in the extract. The coupling experiments were repeated four times, and the average bounding yield was found as 68.18 ± 4.20 mg enzyme/g monomer.

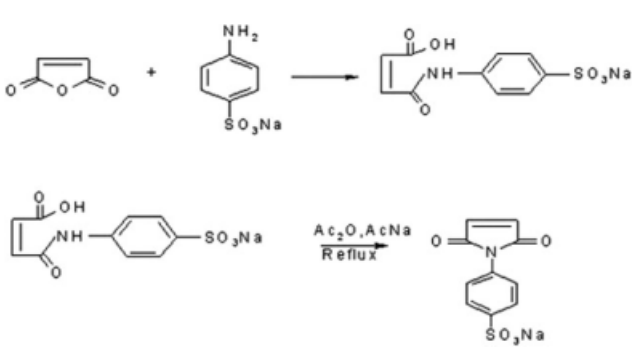
Preparation of enzyme containing UV-cured polymeric films

UV-curable formulation was prepared by mixing 20 wt % enzyme-bounded *N*-(4-sulfophenyl)-maleimide, 31 wt % PEG-diacrylate, 12 wt % PEG-monoacrylate, 34 wt % NVP, and 3 wt % free radical initiator (Irgacure-184). The formulation was poured into the Teflon™ mold, which was cylindrical in shape ($R = 4$ mm). In order to prevent the inhibiting effect of oxygen, the mixture in the mold was covered with a transparent 15-μm-thick Teflon™ film before irradiation with a high-pressure UV lamp, and a quartz glass plate was placed over. The Teflon film was also used to obtain a smooth surface. After 240 s irradiation under UV lamp, 1-mm-thick free polymeric films were obtained. Immobilized enzymes were stored at 4°C until use.

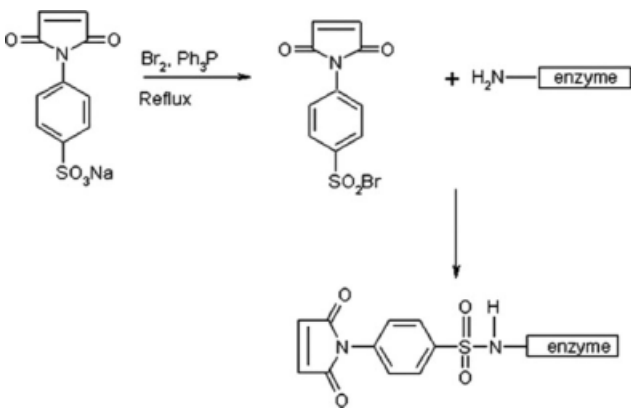
Analysis of the double bond conversion

The degree of double bond conversion of UV-curable formulation was obtained via real-time IR spectroscopy data. The real-time IR technique measures the conversion of double bonds by following the decrease upon UV exposure of the band at 1635 cm^{-1} associated with C=C double bond stretch. The UV curable formulation was coated onto KBr discs in the usual way. The infrared spectrum of the nonirradiated material was recorded, and then wet film was subjected to UV exposure. The IR spectra of the films after UV-curing were taken and the percentage conversion was calculated from the ratio of the corresponding IR absorbance before and after UV exposure (A_0 and A_t) by using the following equation:

$$\text{Conversion } \% = 100(1 - (A_t/A_0))$$



Scheme 1 Synthesis of *N*-(4-sodiumsulfophenyl)-maleimide.



Scheme 2 Preparation of the enzyme bounded monomer.

Activity assay of immobilized and free α-amylase

α-Amylase activity was measured according to the assay suggested by Bernfield.[15] 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. Then, in a test vial, a known amount of polymeric support containing α-amylase was placed. Subsequently 0.5 mL starch solution was added, and the system was incubated in a water bath with constant shaking 100 rpm at 30°C for exactly 5 min. The reaction was stopped by adding 1 mL of DNSA reagent. Incubation was also performed in a boiling water bath for 5 min. After dilution of the reaction content with distilled water, the amount of reducing sugar (maltose) produced was determined spectrophotometrically at 540 nm. In each set of experiments, a standard curve was prepared with maltose solution of different concentrations. Each result is an average of three assays. Activity of the enzyme is calculated from the following equation:

$$\text{Activity (IU mg}^{-1}) = \frac{\text{released maltose (}\mu\text{ mol)}}{\text{amount of }\alpha - \text{amlylase (mg)} \times 5\,\text{min}}$$

The immobilized enzyme had to be removed by washing 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 5.5–8. The results for dependence on pH, storage, temperature, and repeated use are presented in a normalized form with the highest value of each set being assigned as the value of 100% activity.

RESULTS AND DISCUSSION

Preparation and characterization of polymeric support

In this study, α-amylase was immobilized onto a new polymeric support material, which could be a good candidate for rigid support systems. For this purpose, UV-curable *N*-(4-sodiumsulfophenyl)-maleimide monomer was synthesized from the reaction of maleic anhydride and *p*-aminosulfonic acid. Figure 1 shows the FTIR spectrum of the *N*-(4-sodiumsulfophenyl)-maleimide monomer. The peaks at 1778 and 1706 cm^{-1} (C=O in a five-member imide ring) indicate the existence of maleimide functionality.[16,17] The monomer was further characterized by ^{13}C-NMR spectroscopy (Fig. 2). The ^{13}C-NMR spectrum shows the signal of carbonyl carbon of the imide structure at 170 ppm.[18] This evidence demonstrates that the *N*-(4-sodiumsulfophenyl)-maleimide was prepared successfully. Scheme 1 depicts the synthesis route of the *N*-(4-sodiumsulfophenyl)-maleimide monomer.

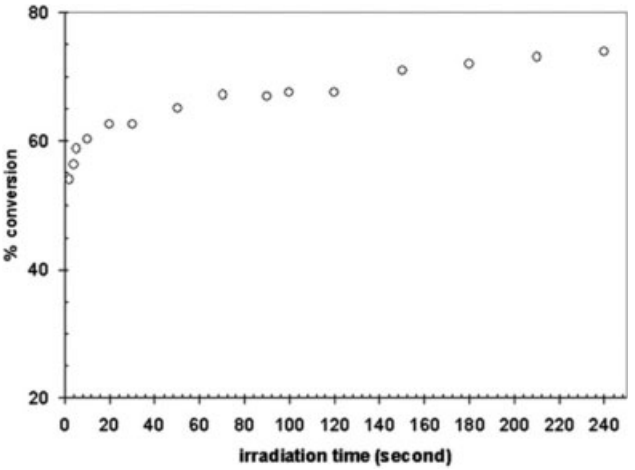


Figure 3 FTIR cure profile for polymeric support.

Afterwards, *N*-(4-sodiumsulfophenyl)-maleimide monomer was reacted with bromine to provide reactive sulfonyl bromide groups for covalent biomolecule immobilization under very mild conditions (0°C, 30 min). As shown in Scheme 2, the covalent coupling of α-amylase to *N*-(4-bromosulfophenyl)-maleimide was achieved by the formation of sulfon amide bonds between the reaction of amino groups of the enzyme and the sulfonyl bromide groups of the maleimide monomer. The enzyme carried maleimide monomer, then solidified quickly by UV-curing technique. It is well known that the fast curing causes less damage to the catalytic activity of enzyme.[19]

Figure 3 shows the conversion curve as a function of irradiation time. As can be seen in Figure 3, the conversion of double bonds reached ~ 74% within 4 min. Schwalm et al. showed that flexibility of UV-cured clear coats is inversely proportional to the coating crosslink density.[20] Right along with this, according to Seubert and Nichols, cure conditions directly influence the acrylate double bond conversion and extend of conversion directly controls crosslink density, too.[21]

Gel content of the UV-cured polymeric film was determined by Soxhlet extraction method for 6 h using pure acetone. Insoluble gel fraction was dried in vacuum oven at 40°C to constant weight, and then the gel content was calculated. Gel content of polymeric film was found to be between 97 and 98%. Swelling was found to be between 0.8 and 0.9%. Because it has very low water absorption capacity and negligible shrinkage behavior, it can be assumed as a very good candidate for a rigid enzyme carrier.[10]

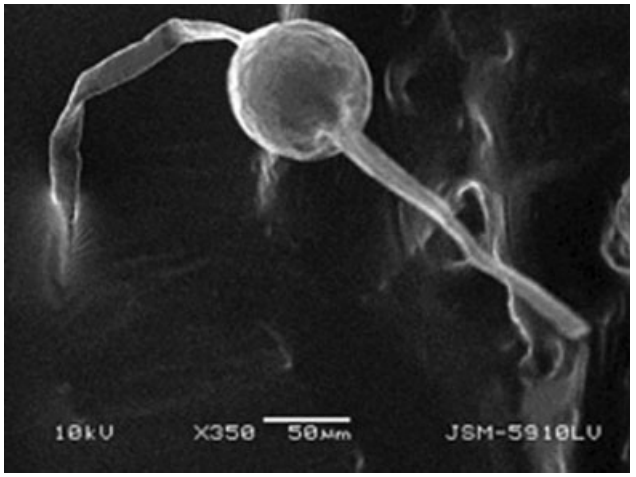


Figure 4 SEM images of the polymeric support at different magnifications.

Surface morphology of the enzyme immobilized polymeric support can be seen in Figure 4. SEM image was obtained by using a JEOL JSM-5919LV SEM, after applying a gold coating under reduced pressure. As seen in Figure 4, a spheroidal shaped particle of about 50 μm in size connected to the polymer via its long arms.

SEM EDS was used to identify the elemental composition of fracture surface of rigid support. EDS analysis revealed that the spherical particles are composed of 55% C, 7% N, and 38% O.

Immobilization efficiency

There are several studies including α-amylase immobilization in which the bonding capacity of the support materials is labile due to the characteristic properties of the prepared materials. In our previous study,[11] the coupling capacity of the α-amylase immobilized onto amine functionalized glass beads was reported as 25.2 mg/g glass support. Pandya et al. studied activity and stability of immobilized α-amylase in ordered mesoporous silicas; the amount of bonded enzyme was reported as 0.5–1.75 mg/g particle.[7] In this study, the amount of the covalently bonded enzyme was found to be as 68.18 ± 4.20 mg/g monomer.

Effect of coupling time on the covalent attachment of enzyme

The effect of coupling time on the covalent attachment of enzyme can be seen in Figure 5. As shown in Figure 5, 59 mg enzyme per gram of monomer, covalently bonded in 8 min. It is easy to observe that α-amylase was covalently bonded onto monomer via coupling reaction between the sulfonyl bromide and the amine functionalities of the enzyme.

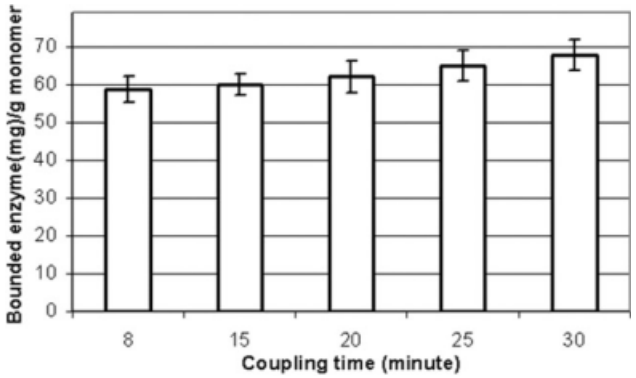


Figure 5 Effect of coupling time on the covalent attachment of enzyme.

An increase in coupling time led to a slight increase in the immobilization capacity. The maximum α-amylase immobilization capacity of monomer was found to be as 68.18 ± 4.20 mg/g monomer.

Influence of pH on enzyme activity

The effect of pH on free and immobilized α-amylase was investigated in buffer solutions in the range of pH 5.5–8.0 at 30°C. Figure 6 shows the dependence of enzyme activity on pH. Both free and immobilized enzymes are sensitive to the pH; however, immobilization did not change the pH-responsibility of the enzyme. The maximum activity was observed at pH 6.5. The pH profile of the immobilized enzyme was broadener than the free enzyme, which means that this method preserves the enzyme activity in a wider pH range. Similar results have been reported by other researchers.[21,22] They compared the activity of soluble and immobilized enzyme as a function of pH and found that both the free and bounded α-amylase had optimum activity at the same pH. This may also be due to diffusional limitations or secondary interactions between the enzyme and the support.[23]

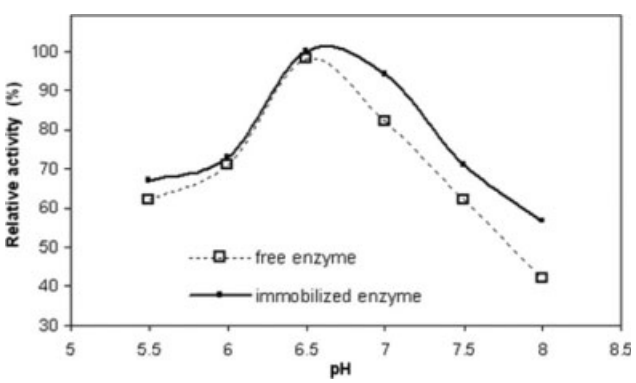


Figure 6 Effect of pH on the relative activity of free and immobilized enzymes.

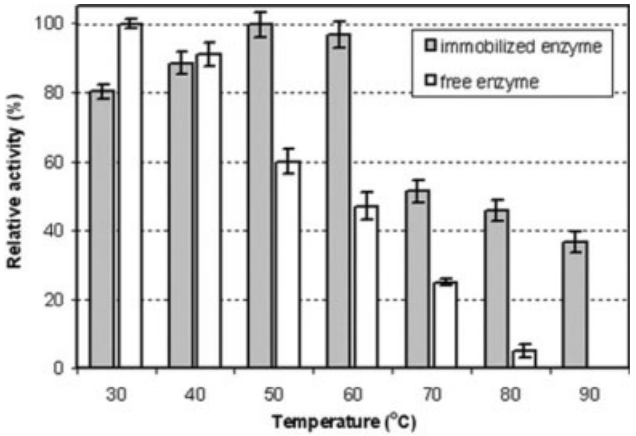


Figure 7 Influence of temperature on the relative activity of free and immobilized α-amylase.

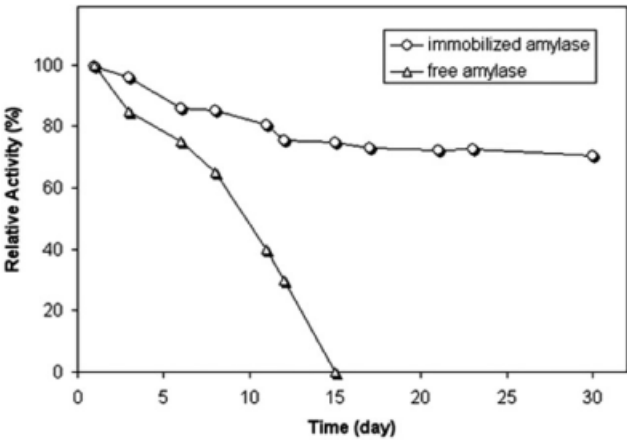


Figure 9 Storage stability of the free and immobilized α-amylase.

Effect of temperature on activity

The thermal stability of immobilized enzymes is one of the most important criteria with regard to their applications. As it is well known, immobilized enzymes, especially in covalently bound systems, are more resistant against heat and denaturing agents than the soluble forms.[24] The temperature dependences of the free and immobilized α-amylase activity was studied in the temperature range of 30–90°C. The effect of the heat treatment on the activity is shown in Figure 7. It was found that the optimum temperature for the free enzyme was approximately 30°C, whereas it shifted to nearly 50°C for immobilized enzyme. The limitation on the enzyme movement can be attributed to covalent bonds between the enzyme and the supports or a low restriction in the diffusion of the substrate at high temperature. Thus, the immobilized enzymes showed their catalytic activities at a higher temperature.[25]

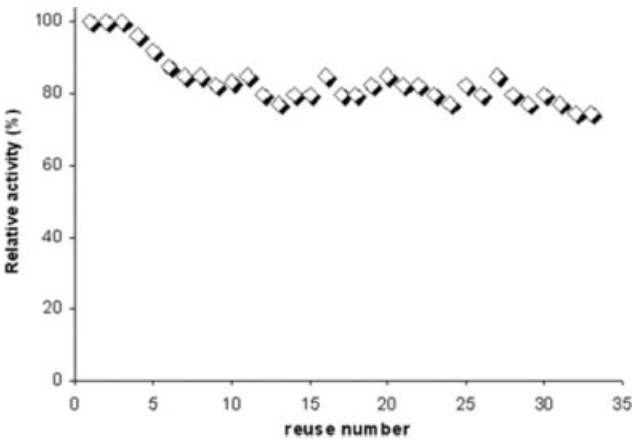


Figure 8 Reuse capacity of the immobilized enzyme.

Reusability

The use of a relatively expensive catalyst as an enzyme, in many instances, requires its recovery and reuse to make an economically feasible process. Immobilization allows ease of removal from stream, reuse of the enzyme, and lowers cost. Reusability experiments were performed at 30°C. After incubation, immobilized enzyme was removed from the reaction medium and washed twice with distilled water. Activity was determined in the same manner as in enzyme assay. To determine the reusability of the immobilized enzyme, the activity of the same support sample was measured sequentially 33 times within a day. The activity found for each repetition was compared with the initial activity, assuming it possesses 100% activity. The decrease in activity after repeated use was given in Figure 8. It was measured that immobilized enzyme exhibited 85% activity after 20 runs. The immobilized enzyme had excellent operational stability, the activity of which remained above 75% of the initial state after 33rd use, whereas free enzyme can be used only once. In our previous study, it was found that the reuse capabilities of α-amylase were in the range of 74–88% when immobilized on epoxidized soybean-based support.[10]

Storage stability

Enzymes are generally not stable during storage in solution, and their activities decrease gradually over time. Immobilized enzymes are more stable than free enzymes.[26] The free and immobilized enzyme were stored in phosphate buffer (0.02*M*, pH 6.9) at 4°C, and their activities were tested for 30 days. Free enzyme lost its activity completely within the 15 days. In Figure 9, it was clearly seen that

immobilized enzyme lost only ~ 30% of its activity at the end of 30 days. This result showed that, by immobilization, enzyme gained more stable character than the free one.

CONCLUSIONS

Polymeric support was prepared by UV-curing technique. Covalent immobilization was performed under very mild conditions and rapidly. The immobilized enzyme is more stable than the native enzyme at higher temperatures. The optimum pH of the immobilized enzyme did not change. The immobilized enzyme had excellent operational stability. It was found that thermal stability and storage stability of immobilized amylase were greater than that of free amylase.

References

1. Mateo, C.; Palomo, J. M.; Fernandez-Lorente, G.; Guisan, J. M.; Fernandez-Lafuente, R. Enzyme Microb Technol 2007, 40, 1454.
2. Monsan, P.; Combes, D. Methods Enzymol 1988, 137, 584.
3. Bergogne, L.; Fennouh, S.; Guyon, S.; Livage, J.; Roux, C. Mol Cryst Liq Cryst 2000, 354, 667.
4. Mateo, C.; Torres, R.; Fernandez-Lorente, G.; Ortiz, C.; Fuentes, M.; Hidalgo, A.; Lopez-Gallego, F.; Abian, O.; Palomo, J. M.; Betancor, L.; Pessela, B. C. C.; Guisan, J. M. Biomacromolecules 2003, 4, 772.
5. Drevon, G. F.; Danielmeier, K.; Federspiel, W.; Stolz, D. B.; Wicks, D. A.; Yu, P. C.; Russell, A. J. Biotechnol Bioeng 2002, 79, 785.
6. Hasirci, N.; Aksoy, S.; Tumturk, H. React Funct Polym 2006, 66, 1546.
7. Pandya, P. H.; Jasra, R. V.; Newalkar, B. L.; Bhatt, P. N. Microporous Mesoporous Mater 2005, 77, 67.
8. Tümtürk, H.; Aksoy, S.; Hasirci, N. Food Chem 2000, 68, 259.
9. Türünç, O.; Kahraman, M. V.; Akdemir, Z. S.; Apohan, N. K.; Güngör, A. Food Chem 2009, 112, 992.
10. Kahraman, M. V.; Ogan, A.; Kayaman Apohan, N.; Gungor, A. J Appl Polym Sci 2006, 100, 4757.
11. Kahraman, M. V.; Bayramoglu, G.; Kayaman Apohan, N.; Güngör, A. Food Chem 2007, 104, 1385.
12. Turner, S. R.; Bridgewater, N. J.; Walker, T. O.; Humble, T.; Warren, A. U.S. Pat. 4,478,727 (1984).
13. Kataoka, T.; Iwama, T.; Setta, T.; Takagi, A. Synthesis (Stuttgart) 1998, 4, 423.
14. Bradford, M. M. Anal Biochem 1976, 72, 248.
15. Bernfield, P. In Advances in Enzymology XII; Nord, F., Ed.; Interscience Publication: New York, 1951; p 379.
16. Park, J. O.; Jang, S. H. J Polym Sci Part A: Polym Chem 1992, 30, 723.
17. Oishi, T.; Fujimoto, M. J Polym Sci Part A: Polym Chem 1992, 30, 1821.
18. Hocking, M. B.; Syme, D. T.; Axelson, D. E.; Michaelian, K. H. J Polym Sci Part A: Polym Chem 1990, 28, 2949.
19. He, D.; Cai, Y.; Wei, W.; Nie, L.; Yao, S. Biochem Eng J 2000, 6, 7.
20. Schwalm, R.; HaÉussling, L.; Reich , W.; Besk, E.; Enenkel, P.; Menzel, K. Prog Org Coat 1997, 32, 191.
21. Seubert, C. M.; Nichols, M. E. Prog Org Coat 2004, 49, 218.
22. Tanyolac, D.; Yuruksoy, B. I.; Ozdural, A. R. Biochem Eng J 1998, 2, 179.
23. Arica, M. Y.; Hasirci, V. J Chem Technol Biotechnol 1993, 58, 287.
24. Chen, G.; Van Der Does, L.; Bantjes, A. J Appl Polym Sci 1993, 48, 1189.
25. Ye, P.; Xu, Z. K.; Che, A. F.; Wu, J.; Seta, F. Biomaterials 2005, 26, 6394.
26. Amaya-Delgado, L.; Hidalgo, M. E.; Montes-Horcasitas, M. C. Food Chem 2006, 99, 299.