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Covalent immobilization of invertase onto the surface-modified polyaniline from graft copolymerization with acrylic acid

Yongjun Chen [a], E.T. Kang [a,*], K.G. Neoh [a], K.L. Tan [b]

[a] *Department of Chemical Engineering, National University of Singapore, 10 Kent Ridge Cresent, Kent Ridge, 119260 Singapore*
[b] *Department of Physics, National University of Singapore, 10 Kent Ridge Cresent, Kent Ridge, 119260 Singapore*

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Abstract

Invertase was covalently immobilized on the emeraldine (EM) base form of polyaniline (PAN) films and powders with surface-grafted acrylic acid (AAc) polymer. The immobilization proceeded via the amide linkage formation between the amino groups of invertase and the carboxyl groups of the grafted AAc polymer chains on EM in the presence of a water-soluble carbodiimide. The surface structure and composition of the grafted–modified and enzyme-functionalized EM base were characterized by X-ray photoelectron spectroscopy (XPS). It was found that the amount of immobilized invertase increased linearly with the concentration of surface-grafted AAc polymer chains. EM powders could be graft-modified and enzyme-functionalized more effectively than EM films. The decrease in activity of the immobilized invertase was considered to be due to, among other factors, the reduced accessibility of substrate molecules to the active sites of the enzyme and the conformational change of the invertase molecules as a result of the covalent immobilization. However, the immobilized enzyme was less sensitive to temperature deactivation below the optimum temperature as compared to that of the free form. The optimum pH value of invertase was not affected by the immobilization reaction, but the pH stability range was broadened. The immobilized invertase also exhibited a significantly improved stability during storage in buffer solution over that of the free enzyme.

Keywords: Polyaniline; Graft copolymerization; X-ray photoelectron spectroscopy; Covalent immobilization; Invertase; Stability

1. Introduction

Enzymes have been utilized in a large number of practical applications, particularly in biomaterials [1,2], bioseparators [3] and biosensors [4], through immobilization on a variety of supports. There are many methods, such as adsorption, entrapment, covalent binding, etc., for enzyme immobilization [5–8]. When the support contains the relevant functional groups, covalent immobilization of enzymes becomes feasible. A range of functional groups which can be used in the covalent immobilization of enzymes include amino, hydroxyl, carboxyl and phenolic groups [9]. The physical structure and chemical composition of support can also influence the microenvironment of the immobilized species and consequently their biological properties [10,11]. Among the various techniques used, surface modification through graft copolymerization with functional monomers appears to be an effective method for introducing specific functional groups on the surface of most polymers for subsequent enzyme immobilization [12]. Enzyme immobilization through covalent bonding has been performed on a number of surface-modified polymers [9,13–16] and recently also on the surface graft copolymerized poly(ethylene terephthalate) (PET)

* Corresponding author. Tel.: +65-874-2189; fax: +65-779-1936.
E-mail address: cheket@nus.edu.sg (E.T. Kang).

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macrofibers [17]. Enzyme immobilization via covalent bonding on electroactive polymers, on the other hand, is less well known [18].

Invertase, as an enzyme, is often used in the food industry because the inversion reaction (hydrolysis of cane sugar to glucose and fructose) can be carried out under mild conditions and there is no coloration problem, unlike the method using common acid hydrolysis. In this regard, the use of immobilized invertase is an attractive option because the re-utilization and the increase in stability of the catalyst can often lead to the cost reduction of the process [19]. The enzyme has been immobilized by different methods on a variety of carriers [5–8]. A number of fine works have been done on the immobilization of invertase on polyethylene [20], copolymer of methacrylamide and *N*,*N*′-methylene bis(acrylamide) [21], and on polypyrrole matrices through entrapment [22]. The electroactive polymer substrates may provide additional advantages over the conventional polymer substrates for enzyme immobilization. The accompanying changes in substrate redox potential and conductivity after the enzymatic reaction may provide additional means for easy sensing and detection of the reactions. Further, the electron-donation ability of electroactive polymer may play an important role in some enzyme-catalyzed reactions, especially in those involving redox reaction.

In this study, the surface modification of electroactive polyaniline (PAN) powders and films via graft copolymerization with acrylic acid (AAc) is first carried out. The surface-modified polymer powders and films are subsequently functionalized through covalent immobilization of invertase. Previous work has revealed that surface graft copolymerization of PAN with protonic acid-containing monomers, such as AAc, readily gives rise to a self-protonated surface structure [12] and a substantial proportion of the grafted protonic acid groups at the surface still remains free for further functionalization, such as the covalent immobilization of enzyme or protein molecules. The surface composition and microstructure of the graft copolymerized and the enzyme-immobilized PAN are characterized by X-ray photoelectron spectroscopy (XPS). The efficiency of surface graft copolymerization, and its subsequent effect on the amount and activity of the immobilized enzyme, are studied in some detail. The relative activity, and the pH, temperature and storage stability of the immobilized and free invertase are also compared.

2. Experimental

2.1. Materials and reagents

The AAc monomer used for graft copolymerization was obtained from Aldrich Chemical Co. of Milwaukee, USA. Water-soluble 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (WSC) was purchased from Dojindo Chemical Co. of Kyoto, Japan, and was used as received. Invertase (Grade V) was supplied by Sigma Chemical Co. of St. Louis, USA. BioRad dye reagent for protein assay (Catalog No. 500-0006) was obtained from BioRad Chemical Co. of Hercules, CA, USA. Dulbecco's phosphate buffer solution (PBS) (containing 8000 mg of sodium chloride, 200 mg of anhydrous monopotassium phosphate in 1 l), used for the enzyme immobilization work, was freshly prepared. The solvents and other reagents were of reagent grade and were used without further purification.

2.2. Polyaniline powders and film

The aniline polymers have the general formula $[(–B–NH–B–NH–)_y(–B–N{=}Q{=}N–)_{1-y}]_x$, in which B and Q denote the C_6H_4 rings in the benzenoid and quinonoid form, respectively. Thus, the intrinsic oxidation states of the polymers can range from that of fully oxidized pernigraniline (PNA, $y = 0$), through that of the 75% intrinsically oxidized nigraniline (NA, $y = 0.25$), the 50% intrinsically oxidized emeraldine (EM, $y = 0.5$), to that of the fully reduced leucoemeraldine (LM, $y = 1$) [23,24].

EM salt of PAN was prepared by the oxidative polymerization of aniline by ammonium persulphate in 1 M HCl according to the method reported in the literature [24]. It was converted to EM base by treatment with excess 0.5 M NaOH. The compensated EM base powders were washed thoroughly with deionized water until it became neutral and then dried by pumping under reduced pressure. Free-standing and lightly cross-linked EM base films of 30 μm thickness were prepared by dissolving EM powders in *N*-methylpyrrolidinone (NMP) (containing 8 wt.% EM base by weight). The solvent was subsequently removed by exhaustive pumping under reduced pressure.

2.3. Surface modification by graft copolymerization

EM powders of about 0.3 g or EM film strips of about 1.0×1.5 cm^2 in area were used in all grafting experiments. The procedures for the surface modification of EM films [12] and powders [25] are similar to those reported earlier. In the case of graft copolymerization with AAc, EM powders or EM films were immersed in an aqueous AAc solution of a predetermined concentration between 0.8 and 15 wt.% in a Pyrex® flask. The reaction mixture was thoroughly purged with N_2 and the flask was subsequently sealed with a ground-glass stopper. The reaction mixture was then kept in a 90°C water bath for about 30 min. After each grafting experiment, the EM powders were removed from the reaction mixture by centrifugation and the EM films were removed from the reaction mixture by a forceps. EM

powders and films were then immersed in a 55°C water bath with continuous stirring for 8–10 h to remove the residual homopolymer. The EM powders were subsequently washed at least six times and were recovered each time by centrifugation. Finally, the EM powders and films were dried by pumping under reduced pressure.

2.4. Immobilization of invertase on the surface-modified EM base powders and films

The method of invertase immobilization used in this work is similar to that reported for the immobilization of other enzyme, such as trypsin, on conventional polymer substrates [17]. The coupling process is shown schematically in Fig. 1. For the covalent immobilization of invertase onto the AAc graft copolymerized EM powders or films, the COOH groups of the grafted AAc polymer were preactivated for 1 h with WSC at 4°C in 0.1 M PBS, containing 5 mg ml^{-1} of WSC. The polymer powders or films were then transferred to 0.1 M PBS(+) (pH 7.4, with 0.02 M $CaCl_2$ added) containing invertase at a concentration of 8 mg ml^{-1}. The immobilization was allowed to proceed at 4°C for 24 h. After that, the reversibly bound invertase was desorbed in copious amounts of PBS(+) for 1 h at 25°C. Physical adsorption of invertase on the EM base was conducted in a similar manner, except that the pretreatment of the powders and films with WSC was omitted.

2.5. Surface characterization after graft copolymerization and after enzyme immobilization

The PAN powders and films after graft copolymerization with AAc and after immobilization of invertase were characterized by XPS. XPS measurements were made on a VG ESCALAB MkII spectrometer with a Mg Kα X-ray source(1253.6 eV photons). The X-ray

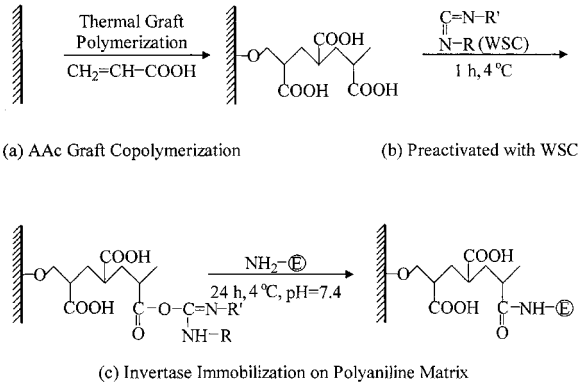

Fig. 1. Schematic diagram illustrating the immobilization of invertase on the acrylic acid (AAc) graft copolymerized EM powder and film surfaces.

was taken at a reduced power of 120 W (12 kV and 10 mA). The polymer powders or films were mounted on the standard VG sample studs by means of double-side adhesive tape. The core-level spectra were obtained at the photoelectron take-off angle of 75°. The pressure in the analysis chamber was maintained at 10^{-8} mbar or lower during each measurement. To compensate for surface charging effects, all binding energies were referenced to the C1s neutral carbon peak at 284.6 eV. In spectral deconvolution, the linewidth (full width at half maximum or FWHM) of Gaussian peaks was maintained constant for all components in a particular spectrum.

2.6. Quantitative determination of the concentration of grafted AAc polymer and the immobilized invertase

The surface concentration of AAc polymer grafted on the EM powders or films was expressed as the weight of the grafted polymer per unit weight of the EM powders or per unit surface area of the EM films, as reported previously [13]. Toluidine blue O (TB) uptake was used to determine the total concentration of the surface-grafted AAc polymer, in weight per unit weight or per unit surface area. An aqueous solution of 1.25×10^{-4} M TB, adjusted to pH 10 with NaOH, was added to the graft-modified samples. The formation of ionic complexes between the COOH groups of the grafted chains and the cationic dye was allowed to proceed for 5 h at room temperature, followed by rinsing the sample substrates with a NaOH solution (pH 10) to remove the uncomplexed TB molecules. The amount of the grafted AAc polymer was calculated from the difference of the optical density at 633 nm of the original dye (1.25×10^{-4} M) and the supernatant solution obtained by centrifugation for EM powders or the residual uncomplexed TB solution after the EM films had been removed, with the assumption that 1 mol of TB had complexed with 1 mole of carboxyl groups of the AAc polymer [26].

The amount of enzyme immobilized on the EM base powders or films was determined by the modified dye-interaction methods [15,27], using the BioRad protein dye reagent. The stock dye solution was diluted five times with double distilled water before use. Invertase solution (0.2 ml) of a known concentration was added to 30 ml of the dye solution. The invertase–dye solution was then kept for 10 min and centrifuged at 5000 rpm for 15 min. In the latter process, the invertase–dye complexes were precipitated and the free dye remained in the upper layer. The absorbance of the supernatant at 465 nm was used for the standard calibration. For the quantitative determination of immobilized invertase, the dye solution (30 ml for EM powders or 20 ml for EM films) was added to a test tube and the invertase-immobilized EM powders (8 mg) or EM films

(1×1.5 cm^2) were immersed in the dye solution. After 3 h of reaction, the powders or films were removed and the absorbance of the dye solution was measured at 465 nm. The amount of invertase immobilized on the surface of EM powders or films was calculated from the standard calibration curve.

2.7. Assay of invertase activity

The enzyme activity of the free and immobilized invertase was measured using the method specified in the Food Chem. Cod [28]. The method is based on the 30 min hydrolysis of sucrose at 30 ± 0.1°C and at pH 4.62. The degree of hydrolysis is determined by measuring the optical rotation of the sucrose solution with a polarimeter. To the flasks containing 20 ml each of the test sample or of the blank, 5 ml of acetate buffer (pH 4.62 ± 0.05) was added. The flasks containing the test sample and the test blank were placed in a circulating water bath maintained at 30.0 ± 0.1°C. The samples were equilibrated for 10 min in the water bath. Twenty five ml of similarly equilibrated sucrose substrate solution (0.24 M) was rapidly pipetted into the test flasks. The incubation period was maintained for an additional 30 min under constant stirring. The reaction was terminated by adding 10 ml of sodium carbonate solution (0.5 M). The flasks containing the test samples and the blank were subsequently placed in a water bath maintained at 20.0 ± 0.1°C for 30 min. For an accurate determination of the results, a polarimeter (Perkin–Elmer 241MC) with an accuracy of at least 0.001 deg of arc was used. The optical rotation of each solution at 589 nm (sodium lamp) line was measured using a 10 cm pathlength optical cell and with the thermostat set at 20.0 ± 0.1°C. The blank run was carried out using the double-distilled water. The above-mentioned activity assays were also carried in the pH range of 3–8 and the temperature range of 20–80°C to determine the pH and temperature activity profile for the free and immobilized enzymes.

3. Results and discussion

3.1. Surface graft copolymerization with acrylic acid

Previous XPS studies [12,23,29] have shown that the quinonoid imine (=N– structure), benzenoid amine (–NH– structure) and positively charged nitrogen in a PAN complex correspond, respectively, to peak components with binding energies (BEs) at about 398.2, 399.4 and >400 eV in the properly curve-fitted N 1s core-level spectrum. Fig. 2(a) and (b) shows, respectively, the C 1s and N 1s core-level spectra of the EM base powders used in this work. The EM base consists of about equal amounts of imine and amine nitrogens,

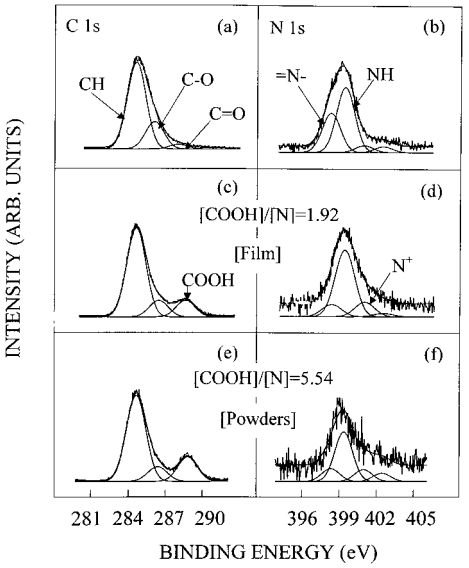

Fig. 2. C 1s and N 1s core-level spectra, obtained at $\alpha = 75°$, of (a) and (b) pristine EM powders, and of (c) and (d) EM base film and (e) and (f) EM powders after having been subjected to thermal graft copolymerization in 12.5 vol% AAc solution.

consistent with the intrinsic redox state of the EM base. The residual high-BE tail above 400 eV in the N 1s core-level spectrum may have resulted at least in part from surface oxidation products or weak charge transfer complexed oxygen [23], as well as from interchain hydrogen bonding in the thermally induced PAN aggregates [30].

Fig. 2(c) and (e) shows the C 1s core-level spectra of the EM powders and the EM film after thermal graft copolymerization in 12.5 wt.% AAc monomer solution. The small but distinct high-BE C 1s component at about 288.7 eV is characteristic of the carboxylic acid group of the grafted AAc polymer [12]. The appearance of this component thus indicates clearly the presence of surface-grafted AAc polymer. The amount of AAc polymer grafted on the surface can be readily determined from the corrected area ratio of the C 1s peak component at 288.7 eV and the total N 1s area, or the [COOH]/[N] ratio. Comparison of the –COOH peak component intensities in Fig. 2(c) and (e) suggests the presence of a higher graft concentration on the EM powders than on the EM film. The phenomenon can probably be attributed to the higher specific surface area of the powder samples. The enhanced N 1s high-BE tail above 400 eV in Fig. 2(d) and (f) and the reduced imine component intensity at 398.2 eV indicate that graft copolymerization with AAc readily results in a self-protonated EM surface with protonation occurring preferentially at the imine units. A significant proportion of the imine nitrogen in these samples, however, remains unprotonated. Thus, steric hindrance and the spatial distribution of

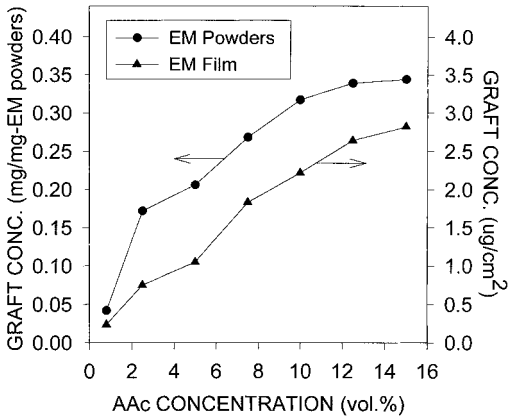

Fig. 3. Effect of monomer concentration on the amount of surface grafted AAc polymer.

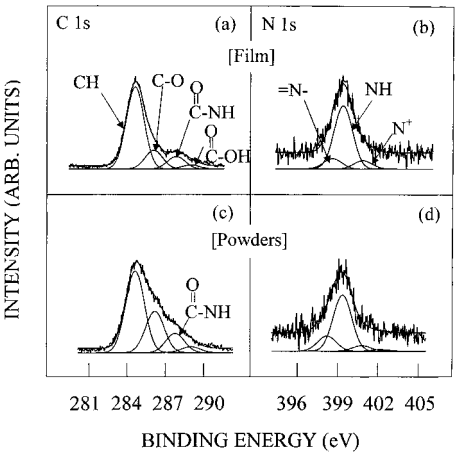

Fig. 4. C 1s and N 1s core-level spectra, obtained at $\alpha = 75°$, of the AAc graft copolymerized (a) and (b) EM films, and (c) and (d) EM powders, after immobilization of invertase.

the grafted AAc polymer must have played an important role in the protonation of EM surface, as already stated in the earlier study [26]. Graft copolymerization with the hydrophilic monomer has also given rise to a very hydrophilic EM surface. For EM films, the decrease in surface water contact angle after graft copolymerization with AAc has been reported in the earlier study [12].

The [COOH]/[N] ratios derived from the XPS results stated above become less accurate at high surface coverage of the AAc polymer. Thus, the concentration of the surface-grafted AAc polymer as a function of the monomer concentration used during thermal graft copolymerization is also determined from the amount of toluidine blue O (TB) uptake [13]. The results are shown in Fig. 3. In general, the concentration of the grafted AAc polymer increases with increasing monomer concentration in the reaction media. In Fig. 3, the higher extent of surface graft copolymerization for EM powders than that for EM films is obvious. The result is also consistent with the higher [COOH]/[N] ratio observed for EM powders than the EM film in Fig. 2.

3.2. Immobilization of invertase

After activation of the carboxyl groups of the grafted AAc polymer on the surface of EM base by WSC, invertase was covalently immobilized (see Section 2). Fig. 4 shows the XPS C 1s core-level spectra of the AAc graft copolymerized EM film and powders surfaces with covalently immobilized invertase. In comparison with the corresponding spectra before invertase immobilization (Fig. 2), the intensity of the peak component at the BE of 288.7 eV associated with carboxyl group is reduced, whereas, the intensity of the peak component at the BE of 287.8 eV associated with the peptide linkage (CONH) is substantially enhanced. The above changes in the relative intensities of the components in the C 1s core-level spectra indicate that invertase has been successfully immobilized through amide linkages on the surface of the EM base. The present results are consistent with the scheme generally proposed for the covalent immobilization of proteins or enzyme on the AAc graft copolymerized polymer substrates [9]. In Fig. 4(b), the peak intensity for the peptide linkage (CONH) of the EM powders is significantly higher than that in Fig. 4(a) for the EM film. This result is again consistent with the presence of a higher AAc polymer loading for the EM powders.

The surface concentration of immobilized invertase on the EM base is expressed as the weight of immobilized invertase per unit weight of the EM powders or per unit surface area of the EM film, and is determined using the protein–dye reagent [15,27]. Fig. 5 shows the amounts of covalently immobilized and physically absorbed (in the absence of WSC activation) invertase as a function of the concentration of surface grafted AAc polymer (expressed as the concentration of the AAc monomer used in the graft copolymerization, see also Fig. 3). The covalent coupling between the carboxyl groups of the grafted AAc chains and the invertase molecules through the WSC intermediate results in a linear increase in the amount of immobilized invertase with the concentration of grafted AAc polymer. The maximum amount of immobilized invertase attained was about 1.26 mg per mg of EM powders and 0.19 mg cm^{-2} of the EM film. In contrast, the amount of physically absorbed invertase becomes saturated at a relatively low level, in comparison with that of covalently immobilized invertase in the presence of WSC. The physically adsorbed invertase may have resulted from the ionic interaction between the enzyme and the imine/amine groups of the EM base. Also, the greater amount of the

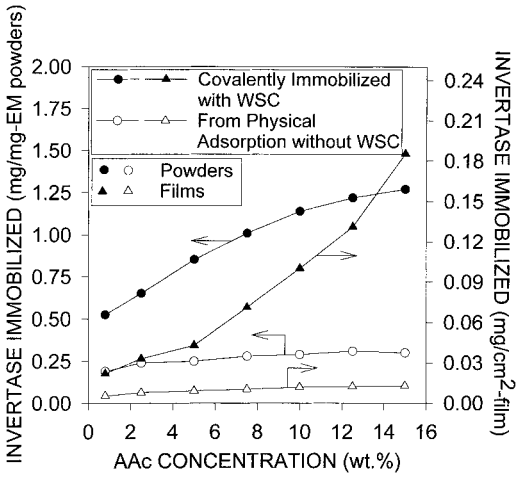

Fig. 5. The amount of covalently immobilized and physically absorbed invertase on the EM base powders and films at different concentrations of grafted AAc polymer (expressed as the AAc monomer concentration used for graft copolymerization).

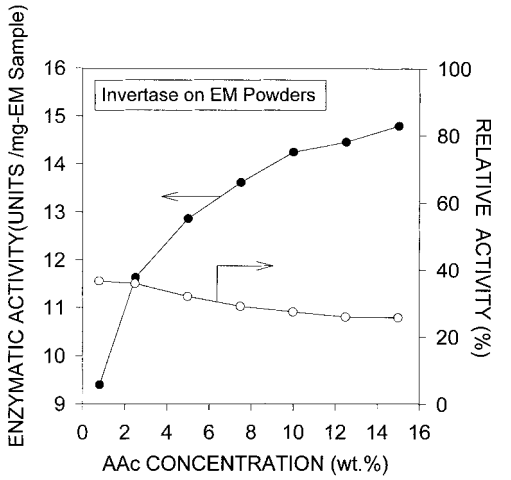

Fig. 6. Equivalent and relative activities of the covalently immobilized invertase on the EM powders as a function of the surface concentrations of grafted AAc polymer.

immobilized invertase on the EM powders suggests that the EM powders are more effective in the immobilization of the enzyme due to their higher level of AAc polymer loading.

The activity of the immobilized invertase, expressed as the quantity of enzyme that would hydrolyze 1.142 μmol of sucrose per min under the condition of assay, and its relative activity (RA), defined as the ratio of the observed activity of the surface immobilized enzyme over the activity obtained from an equivalent amount of the free (native) enzyme, can be assayed with little ambiguity. The surface enzyme activities as a function of the concentration of surface-grafted AAc polymer (in terms of monomer concentration used for graft copolymerization, see also Fig. 3) for the invertase-immobilized EM base powders and film are shown, respectively, in Figs. 6 and 7. At low concentrations of surface-grafted AAc polymer, the observed enzyme activities increase linearly with increasing AAc polymer concentration. This increase in the observed activity must be associated with an increase in the amount of surface-immobilized enzyme. The increase in surface enzyme activities gradually lags behind the increase in the AAc polymer concentration, although the actual amount of the immobilized enzyme continues to increase, as shown in Fig. 5. This phenomenon is also reflected in the decrease in RA of the immobilized enzyme at moderate to high concentrations of grafted AAc polymer in Figs. 6 and 7.

The decrease in the relative enzyme activity at high AAc polymer concentration suggests that in the presence of diffusion limitation, a considerable fraction of the enzyme molecules is probably embedded in the grafted AAc polymer layer and becomes less accessible. Thus, the enzyme at the top surface must have completely hydrolyzed all the sucrose before the latter can reach the invertase immobilized in the subsurface layer of the grafted AAc polymer. A similar conclusion has been put forward for the saturation of enzyme activity for trypsin immobilized on the AAc graft copolymerized PET fibers [17]. Further, the inhibition in enzyme activity at high surface concentration of the AAc polymer can arise from the increase in steric hindrance, decrease in pH value, and increase in interaction between the active sites of the enzyme and the AAc polymer.

Comparison of the data in Figs. 6 and 7 suggests that a significantly higher enzyme activity is observed on the

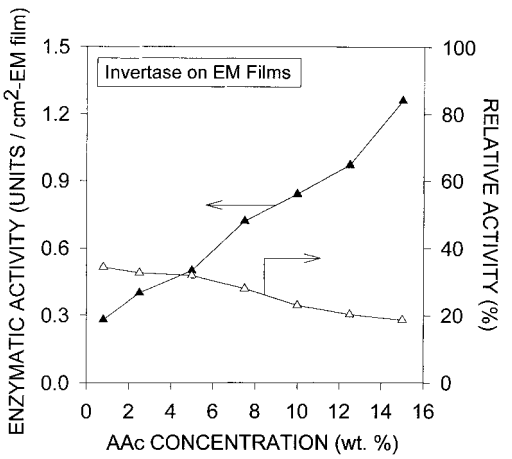

Fig. 7. Equivalent and relative activities of the covalently immobilized invertase on the EM films as a function of the surface concentrations of grafted AAc polymer.

EM powders. Nevertheless, the relative activities of the enzyme on the EM powders and films are comparable in the concentration range of the grafted AAc polymer studied. Further, the invertase immobilized on the EM surface still retains about 20–40% of its activity when compared to that of the free enzyme. Finally, the enzyme activity must have also been affected by the low pH value of the surface, especially, at a high concentration of the surface grafted AAc polymer. The effect of the pH value of environment on the activity of the free and immobilized enzyme will be addressed below (see also Fig. 9).

3.3. Kinetic effect of immobilization

In order to study the kinetic effect of the immobilization, the rates of the sucrose hydrolysis reaction by the native and immobilized invertase were measured at various sucrose concentrations. Fig. 8 shows the Lineweaver–Burk plots for the rates of sucrose hydrolysis by the native invertase and the immobilized invertase on the EM powders. The plots give rise to straight lines which conform to the Michaelis–Menten equation for the enzyme-catalyzed reaction.

The maximum reaction rate V_{max} and the apparent Michaelis constant K_m determined from the linear regression of each plot in Fig. 8 are presented in Table 1. The K_m value for the invertase immobilized on EM base powders is larger than that for the free invertase. The difference in K_m values between the free and the immobilized invertase can be attributed to the limited accessibility of sucrose molecules to the active sites of the immobilized invertase, especially at a high concentration of the grafted AAc polymer, as a result of the spatial distribution of the invertase molecules in the grafted AAc polymer layer and the conformational changes of invertase molecules caused by the covalent immobilization. The decrease in V_{max} value as a result of immobilization is considered to be associated with the increase in K_m value since the lower the value of K_m, the greater the affinity between the enzyme and the substrate.

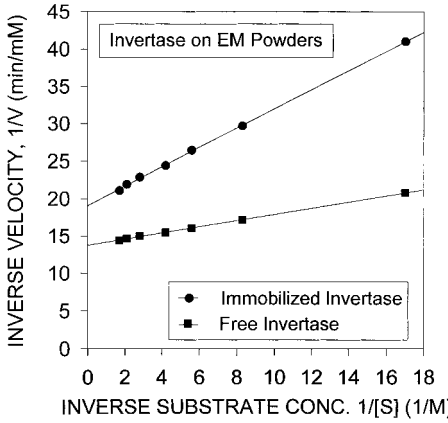

Fig. 8. Lineweaver–Burk plots for the native invertase and immobilized invertase on the EM powders in pH 4.6 acetate buffer at 55°C. (For free invertase: r^2 (regression) = 0.9993; for immobilized invertase: $r^2 = 0.9996$).

Table 1
Kinetic parameters for free and immobilized invertase

Invertase	V_{max} (mM min^{-1})	K_m (mM)
Free	0.073	29.9
Immobilized	0.052	67.6

3.4. Effect of pH and temperature on the activity of immobilized invertase

Enzymes are very fragile molecules when removed from their natural medium. A change in pH, ionic media or temperature may cause denaturation. The pH effect on the activity of the free and immobilized forms of invertase has been studied in buffer solution at different pH values (pH 3–8) and is presented in Fig. 9. The pH values for optimum activity of the free and immobilized invertase are almost the same (around 4.6). However, the pH-dependent activity profile for the immobilized invertase is considerably expanded. Thus, the immobilized invertase displays significantly improved stability over the free form at lower pH values. This result can probably be attributed to the stabilization of invertase molecules resulting from multipoint attachment of the enzyme molecules on the surface of the AAc graft copolymerized EM powders, as well as the diffusional limitations of the immobilized enzyme molecules [31]. The maintenance of optimum pH for the free and immobilized invertase can probably be attributed to the fact that the concentration of the charged species (hydrogen

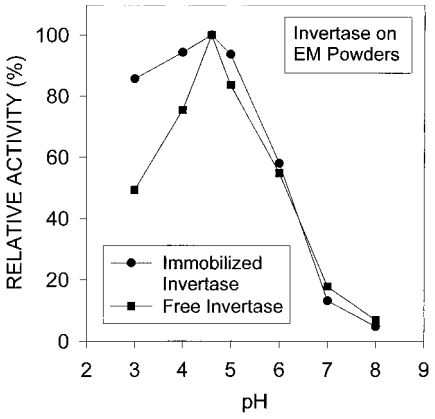

Fig. 9. The relative activity of free invertase and immobilized invertase on the EM powders at different pH values.

ions, for instance) in the domain of the immobilized enzyme is similar to that in the bulk solution [8].

The effect of temperature on the activity of the free and immobilized invertase is shown in Fig. 10. The activity of the invertase is strongly dependent on temperature, with the optimum temperature being observed between 50 and 60°C. However, the activity of the free invertase showed a more critical temperature dependence at temperatures below the optimum temperature than that of the immobilized invertase. The immobilized invertase still retains half of its optimum activity at 20°C. However, the activity of the immobilized invertase shows a more critical temperature dependence at temperatures above the optimum temperature. This phenomenon can probably be partially accounted for by the decrease in the optimum temperature for enzyme activity after immobilization. The free and immobilized invertases retain only less than 10% of their optimum activities at temperature above 70°C. Another plausible explanation for the difference in temperature activity profiles between the free and immobilized invertase is that the latter is less susceptible to temperature-induced conformational changes after covalent immobilization on the AAc graft copolymerized EM powders [31] while the quarternary structure of the free invertase may be easily disaggregated [32]. The immobilization procedure probably helps to maintain the oligometric forms (mainly, octameric and hexameric aggregates) of the enzyme prevailing in the free invertase solution [33]. The denaturation of the immobilized enzyme could take place on the tertiary structure of the peptidic chains of the invertase, which would also occur for the free form after 4 min at 70°C [18]. In other words, as the temperature increases continuously after the optimum temperature the structure of the enzyme becomes altered (denatured) and its catalytic properties are reduced and eventually destroyed.

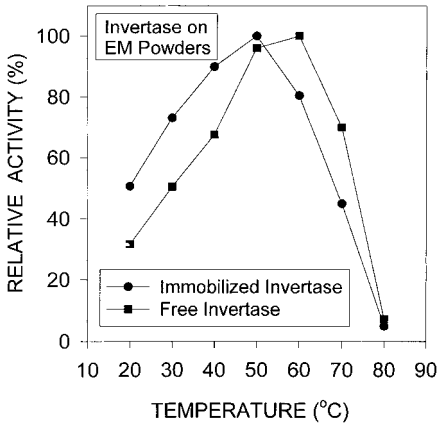

Fig. 10. The effect of temperature on the relative activity of free invertase and immobilized invertase on the EM powders.

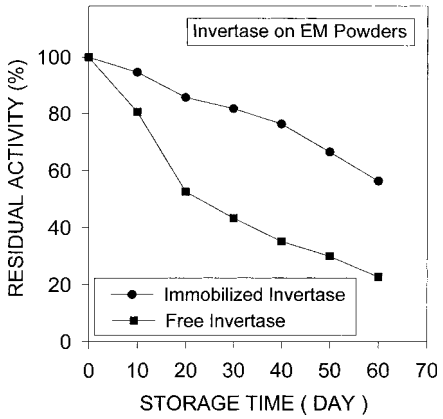

Fig. 11. The storage stability of free invertase and immobilized invertase on EM powders in the pH 7.4 phosphate buffer at 4°C.

3.5. Storage stability of the immobilized invertase

One of the most important parameters to be considered in enzyme immobilization is storage stability. The stability of the immobilized invertase on AAc polymer graft copolymerized EM powders was examined after the immobilization powders were stored in phosphate buffer solution (pH 7.4) at 4°C in the dark for a predetermined period of time. Free invertase solutions were also prepared and stored under the same conditions. Under the same storage conditions, less reduction in activity for the immobilized invertase is observed. As shown in Fig. 11, the immobilized invertase molecules still retain more than 50% of their original activity in the buffer solution over a period of two months, while the activity of the free invertase molecules has decreased to less than 25% of its original value over the same period of time. This result readily suggests that the immobilized invertase exhibits an improved storage stability over the free enzyme. The improved stability of the immobilized invertase can be attributed to a reduction in the rate of thermal denaturation of the enzyme [34], as a result of covalent fixation of the invertase molecules on the surface of the EM powders.

4. Conclusions

Surface modification of EM powders and films was achieved through thermal graft copolymerization with AAc. It was demonstrated that the so-modified EM base could be further functionalized by covalent immobilization of an enzyme, invertase. The covalently immobilized enzyme still retains 20–40% of its activity when compared to that of the free form. EM powders were significantly more effective for the immobilization of the

invertase than the EM films. The pH value for optimum activity of invertase was not affected by the immobilization reaction. However, the activity of the immobilized invertase was expanded over the lower pH range. Covalent immobilization also helped to improve the activity of the enzyme at low temperatures. The storage stability of the immobilized invertase in buffer solution was also enhanced over that of the free enzyme.

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