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INVERTASE IMMOBILIZATION ONTO RADIATION-INDUCED GRAFT COPOLYMERIZED POLYETHYLENE PELLETS

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Abstract—The graft copolymer poly(ethylene-g-acrylic acid) (LDPE-g-AA) was prepared by radiation-induced graft copolymerization of acrylic acid onto low density polyethylene (LDPE) pellets, and characterized by infrared photoacoustic spectroscopy and scanning electron microscopy (SEM). The presence of the grafted poly(acrylic acid) (PAA) was established. Invertase was immobilized onto the graft polymer and the thermodynamic parameters of the soluble and immobilized enzyme were determined. The Michaelis constant, K_m, and the maximum reaction velocity, V_{max}, were determined for the free and the immobilized invertase. The Michaelis constant, K_m was larger for the immobilized invertase than for the free enzyme, whereas V_{max} was smaller for the immobilized invertase. The thermal stability of the immobilized invertase was higher than that of the free enzyme. Copyright © 1996 Elsevier Science Ltd.

INTRODUCTION

A large variety of inorganic and organic solid supports have been used for chemical immobilization of enzymes (Chen *et al.*, 1993; Hyndman *et al.*, 1992; Onyezeli, 1989). Radiation grafting has contributed to the synthesis of functional polymers which can be used for immobilization of enzymes by the chemical binding method. These can have many advantages, such as efficient fixation of the biocomponent in the matrix and lower inactivation of the enzyme (Gombotz *et al.*, 1985). Several authors have reported on the immobilization of enzymes on polymers obtained by radiation graft polymerization, such as 2-hydroxyethyl methacrylate (Beddows *et al.*, 1988; Da-Silva *et al.*, 1990; Hoffman, 1977).

In countries where the main sources of sugar are beet or cane, inverted sugar syrup, which can be obtained by acid or enzymic hydrolysis, is a valuable commercial product for the food industry (due to its low crystallization rate and high sweetening power), apart from its use as raw material for the production of glucose and fructose.

With acid hydrolysis, the final syrup is often contaminated with coloured oxidation compounds, which arise from cyclization of hexoses at low pH and high temperatures. Such a problem does not occur with invertase, because the inversion reaction (hydrolysis of cane sugar to glucose and fructose) is carried out under mild conditions (high pH and lower temperatures).

Nevertheless, the use of immobilized invertase is an option which may be considered, because the reutilization and increase in the stability of the catalyst can often lead to a cost reduction in the process (Godfrey and Reichett, 1983). This enzyme has been immobilized by different methods (adsorption, entrapment, covalent binding) in a variety of carriers (Onyezili *et al.*, 1981; Ikeda *et al.*, 1986; Nakajima *et al.*, 1989; Vitolo and Barros, 1992).

In this work invertase was immobilized onto low density polyethylene pellets and grafted with poly(acrylic acid) by ionizing radiation. The usefulness of polyethylene pellets as the support for binding the enzyme can be ascribed to: (1) its easy graftability, (2) low cost and (3) easy extraction.

Some thermodynamic parameters such as activation energy, enthalpy, entropy and Gibbs free energy change for the free and immobilized forms of invertase were determined, as part of this work.

EXPERIMENTAL

Materials

Invertase (from *Saccharomyces cerevisiae*) was purchased from Solvay Enzymes Inc. The low density polyethylene (LDPE) pellets (Poliolefinas Co.) of diameter 3 mm were washed with water followed by methanol, filtered and dried to a constant weight. Acrylic acid (AA) was obtained from Aldrich Chemical Co. and was used as received. All the other

reagents were commercially available products of analytical grade.

Grafting procedure

Simultaneous radiation grafting method was used as a technique for the preparation of poly(ethylene-g-acrylic acid) (LDPE-g-AA) pellets. Tared, dry LDPE pellets (10.0 g) were added in water/AA mixture of known (30% w/w) bulk monomer concentration. The LDPE pellets, monomer and solvent were put in a glass ampoule. The ampoule was connected to a vacuum system (0.013 Pa) and it was evacuated by a freeze–thaw cycle which was repeated five times. After evacuation, the ampoule was irradiated by using gamma-rays from a ^{60}Co source at a dose rate of 0.033–0.468 kGy/h, and a total dose of 6.0 kGy. The grafted pellets were washed thoroughly with hot distilled water and soaked in distilled water for 48 h to extract the residual monomer, and the homopolymer, poly(acrylic acid), occluded in the pellets. The pellets were then dried in a desiccator under vacuum at room temperature until a constant weight was reached.

The degree of grafting was determined by the percentage increase in weight as follows:

$$\text{Degree of grafting } (\%) = \frac{W_g - W_0}{W_0} \cdot 100$$

where W_0 and W_g represent the weights of the initial and grafted pellets, respectively.

Surface Characterization

Photoacoustic spectroscopy (PASIR)

The graft copolymers were analysed by Fourier transform infrared photoacoustic spectroscopy (FTIR). The analyses were performed using a Bomen model DA 3.02 spectrophotometer and a photoacustic MTEC accessory model 200. Charcoal was used as reference.

Scanning electron microscopy

For morphological examination, the LDPE and LDPE-g-AA pellets were sputter-coated with gold and observed by a scanning electron microscope (SEM) (JEOL JXA-6400 electron probe microanalyzer).

Water-uptake measurements

The graft copolymer samples (1.0 g) were immersed in distilled and deionized water (10 ml) at 25°C for 24 h. The samples were then filtered and weighed. The samples were then dried to a constant weight under vacuum at room temperature (25°C). The water-uptake percent ($H\%$) was calculated as follows:

$$H(\%) = \frac{(\text{initial wet weight} - \text{final dry weight})}{\text{final dry weight}} \cdot 100$$

Activating carboxyl groups

The activation of the LDPE-g-AA pellets (20% grafting degree) was similar to that described by Coulet and coworkers (Coulet *et al.*, 1974). The dried supports (10 g) were esterified by refluxing the pellets with an excess of methanol (100 ml), containing 1% by weight of sulfuric acid as catalyst, for 24 h. The pellets were washed thoroughly with water and then were immersed in a 2% solution of hydrazine in a nitrogen atmosphere at 25°C for 24 h. The pellets were removed and washed with water. The azide formation was accomplished by immersing pellets in 35 ml of cold 4.3 mol/dm^3 HCl and 1 ml of 3.0 mol/dm^3 aqueous $NaNO_2$. The reaction mixture was swirled for several seconds and the pellets were allowed to stand for 5 min. The LDPE-g-AA activated pellets were removed and washed with cold NaCl 0.1 mol/dm^3.

Enzyme immobilization

The invertase was covalently immobilized on the activated surfaces of LDPE-g-AA pellets. The coupling process is shown in Fig. 1 (step IV). The LDPE-g-AA pellets with activated carboxyl groups are dipped into the invertase solution (0.4 mg/ml, deionized water) at 4°C, for 24 h. This last step secures covalent binding of invertase through their free amino groups to poly(acrylic acid) grafted onto polymeric surfaces with activated carboxyl groups. The pellets were then washed with cold distilled water and stored in deionized water at 4°C. The measurement of the protein content, before and after the immobilization process, was performed by using the Bradford's method (Bradford, 1976).

Determination of Invertase Activity

Free invertase

A standard test for free invertase consisted of mixing 1.0 ml of invertase (previously diluted 2000 times) with 100 ml of sucrose solution (100 g/l in 0.010 mol/dm^3 citrate–phosphate buffer, pH 4.6). The hydrolysis was carried out for 10 min at 37°C under constant stirring (200 revolutions/min). For monitoring the hydrolysis, 0.5 ml samples were taken every 2 min, and transferred to a Folin-Wu test-tube containing 1.0 ml of the first Somogyi reagent (Somogyi, 1952), which was quickly immersed in a boiling water bath for 10 min.

Thereafter, the procedure was followed as described previously (Vitolo and Borzani, 1983). The initial invertase activity (v_0) was calculated (always in duplicate) from the slopes of the total reducing sugars (TRS) vs time of reaction plots (Fig. 2 is an example of such a plot). One invertase unit (U) was defined as the quantity of TRS (mg) formed per min under the conditions of the test. The diluted invertase had an activity equal to 8.64×10^{-3} U/ml, according to this method. The standard deviation at 95% level of confidence was ± 0.0152.

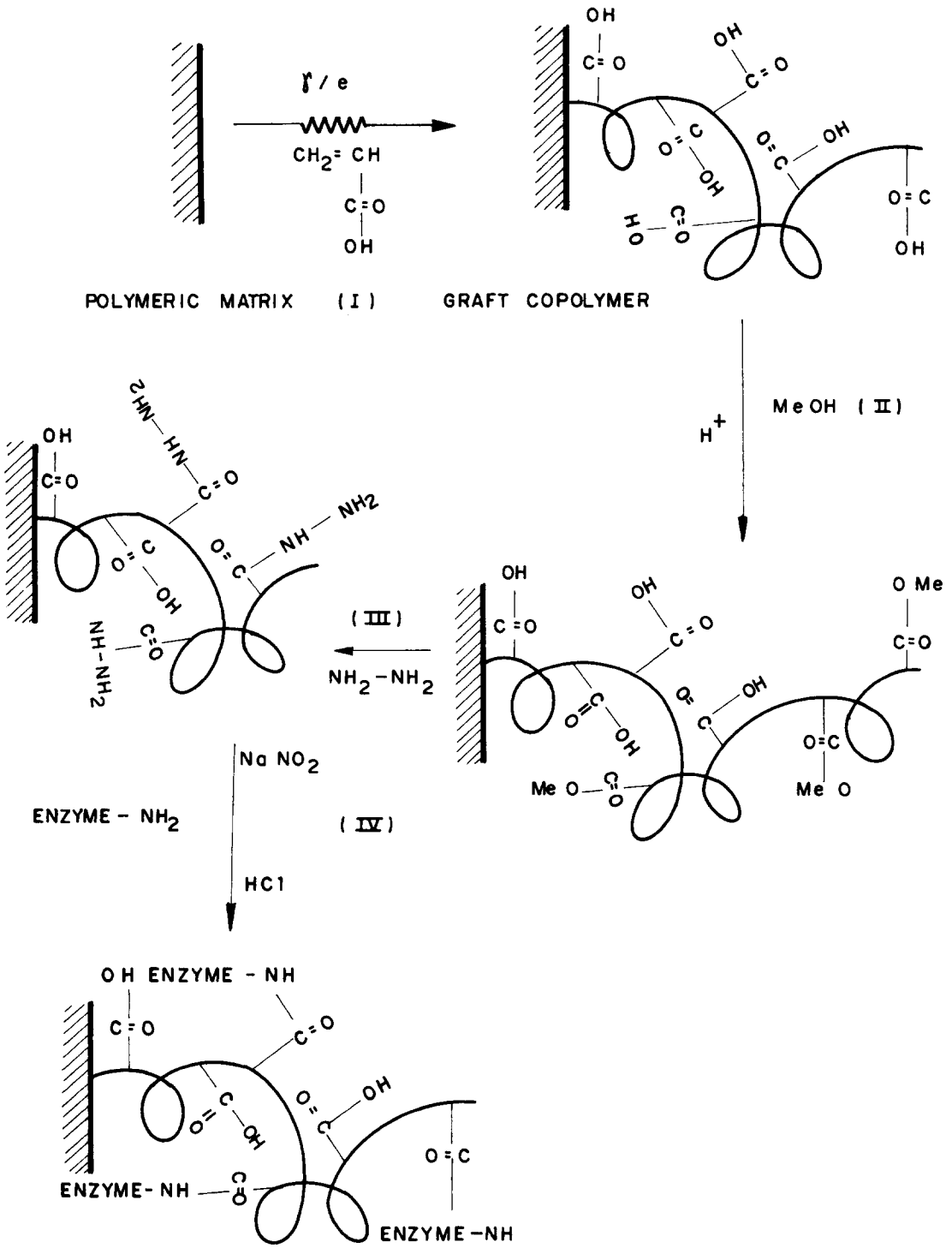

Fig. 1. Schematic diagram of the invertase immobilization onto LDPE pellets functionalized with radiation-induced graft copolymerization of acrylic acid.

Immobilized invertase

The washed LDPE-g-AA-invertase complex (10.0 g) was resuspended in 100 ml of buffered sucrose solution and the reaction carried out for 25 min as already described, except for sampling, which was now 0.5 ml every 5 min. The immobilized invertase had an activity equal to 4.32×10^{-3} U/ml (expressed in accordance with the total reaction volume of 100 ml). The standard deviation for this procedure at 95% level of confidence was ± 0.0212. The immobilization yield was calculated by the ratio of the V_{max} of the immobilized invertase and the free enzyme.

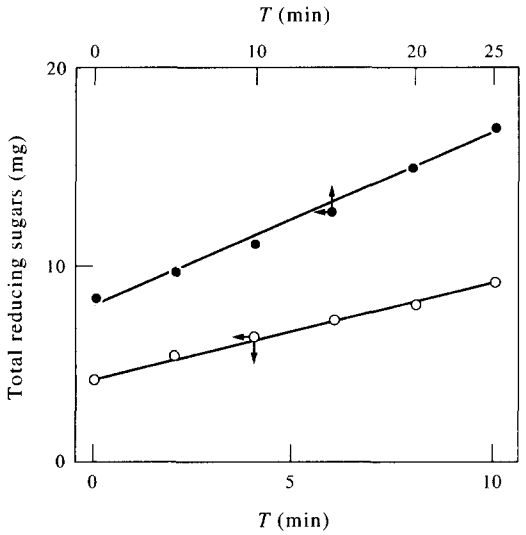

Fig. 2. Formation of reducing sugars from hydrolysis of sucrose by free invertase (○) and invertase immobilized on LDPE-g-AA (●).

Measurement of soluble protein

This determination was done according to Bradford (Bradford, 1976), using bovine serum albumin (Sigma, fraction V powder) as the standard protein.

Effect of pH, sucrose concentration and temperature on the free and immobilized invertase

By changing individually the conditions of the standard test (pH from 2.6–6.8, temperature from 30–70°C and sucrose concentration from 5–50 mmol/dm^3) the following parameters were determined: (1) K_m and V_{max} through the Lineweaver–Burk linear plot method, (2) the effect of pH and temperature on the invertase activity, (3) the activation energies by the Arrhenius method, (4) the stability of both forms of invertase against pH after 15 min of invertase–buffer contact, the mixture being maintained at 37°C and (5) the stability of the free and immobilized invertase against temperature, from the inactivation data. In addition, it was verified that the non-enzymic decomposition of sucrose at different temperatures and pH was negligible and that the total volume of samples withdrawn for testing never exceeded 5% of the total reaction volume. The thermodynamic parameters (ΔH, ΔG, ΔS and E_a) were calculated in accordance with the conventional thermodynamic equations (Atkins, 1987).

Table 1. Influence of grafting degree on water sorption of the LDPE pellets

Grafting degree (%)	Water sorption (%)
0	0.10
5.65	3.15
12.31	9.78
13.57	10.15
20.75	15.78

RESULTS AND DISCUSSION

Water sorption

In order to investigate the hydrophilicity of the graft copolymer, the pellets of LDPE-g-AA were kept in water for 24 h at 25°C, filtered and re-weighed. The results (Table 1) show that the water sorption increased with increase in the degree of grafting. The hydrophilicity of LDPE is improved because the hydrophilic–COOH groups were added to it by radiation grafting.

Photoacoustic spectroscopy

Figure 3 shows the photoacoustic infrared spectrum of the copolymer with 20% grafting degree. As might be expected, the infrared spectra of the copolymer showed a characteristic absorption peak of the carbonyl group at 1719 cm^{-1}. The broad band at 3000 cm^{-1} can be assigned to the stretching mode of the hydrogen-bonded hydroxyl of the grafted PAA. The PASIR spectrum of the LDPE-g-AA demonstrates that the intensity of the vibrational modes of the CH_2 bands (730, 1370 and 2900 cm^{-1}) of the LDPE were reduced, indicating the grafting of the PAA onto the LDPE pellets.

Scanning electron microscopy

The morphology of the samples was investigated by SEM (Fig. 4). The LDPE virgin pellets show a spherulitic texture [Fig. 4(a)]. The surface morphology of the graft copolymers LDPE-g-AA is quite different from the original LDPE pellets. The increase of the grafting level reveals more complex structures [Fig. 4(b, c)]. This may be associated with a homogeneous distribution of the poly(acrylic acid) (PAA) grafted chains at the higher levels of grafting. Furthermore, the increased roughness with the increased grafting degree may be due to the collapse of the gel structure onto the pellet surface.

Properties of the Immobilized Invertase

Immobilization yields

The invertase immobilization process described in this work gave a coupling yield of 98%. However, the activity of the immobilized invertase was around 50%. This may be due to interactions between the active site of the enzyme with the functional groups present on the polymeric support.

Activity versus pH

The effect of pH on the activity of the free and immobilized enzyme is given in Fig. 5. The optimum pH for both the enzyme forms (free and immobilized) was 4.6, although the enzyme activity decreased with the immobilization procedure, 11.6×10^{-3} U/ml to 2.9×10^{-3} U/ml for free and immobilized forms, respectively.

The maintenance of optimum pH for the free and immobilized invertase could be attributed to the fact that the concentrations of charged species (hydrogen

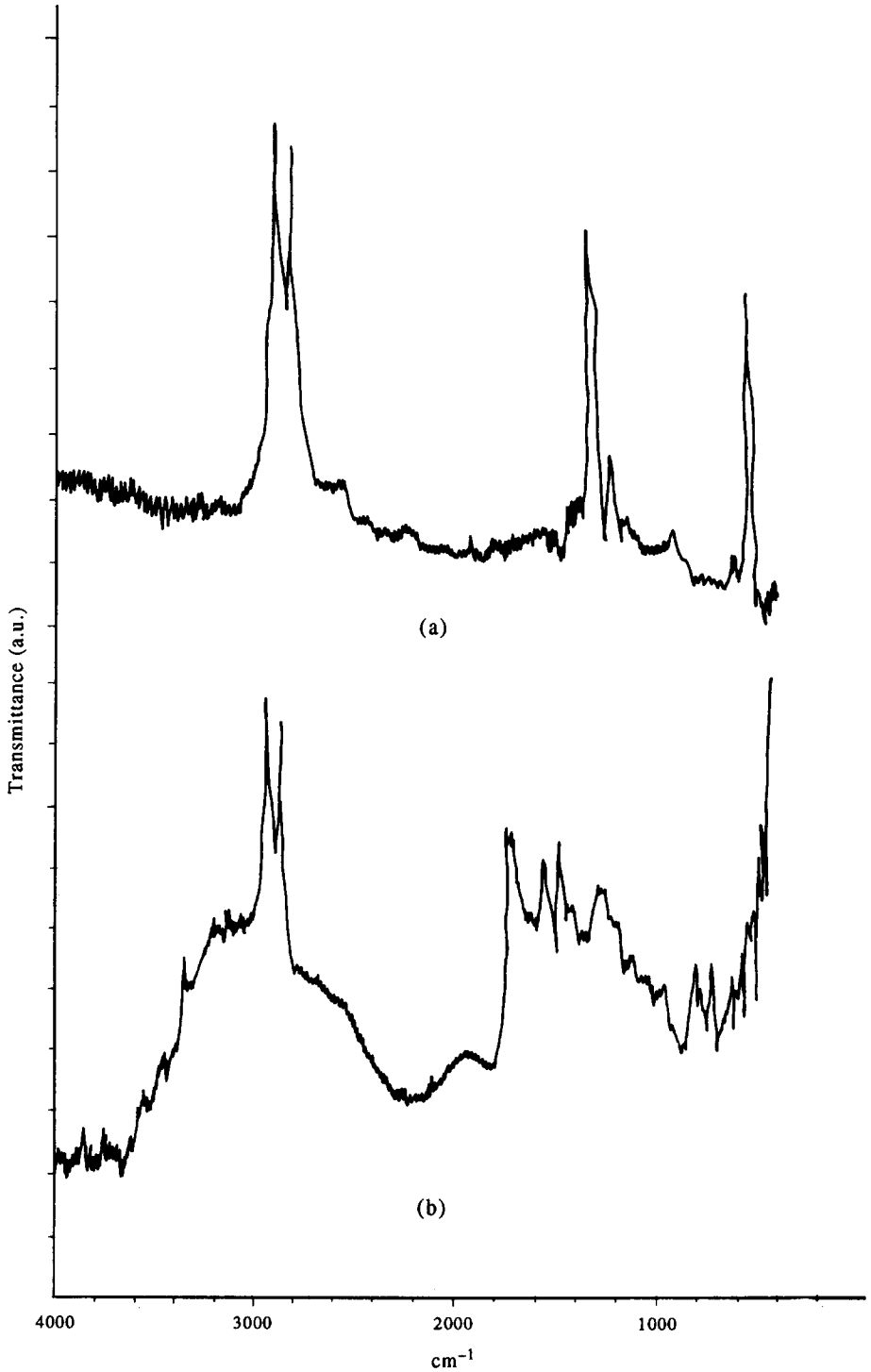

Fig. 3. Photoacoustic FT-IR of the (a) LDPE pellets and (b) LDPE-g-AA pellets. *G*: 20%.

ions, for instance) in the domain of the immobilized enzyme are similar to that in the bulk solution (Vitolo and Barros, 1992). From Fig. 5 we can also see that immobilization favoured the stabilization of invertase against pH variations, since the immobilized and free invertases were stable between 4.0–5.0 and 3.2–5.5, respectively.

Stability against Temperature

As can be seen from Fig. 6, the immobilized invertase showed high thermal stability compared to the free form. The inflection points observed for the free form may be associated with the disaggregation of the invertase quaternary structure (Esmon *et al.*,

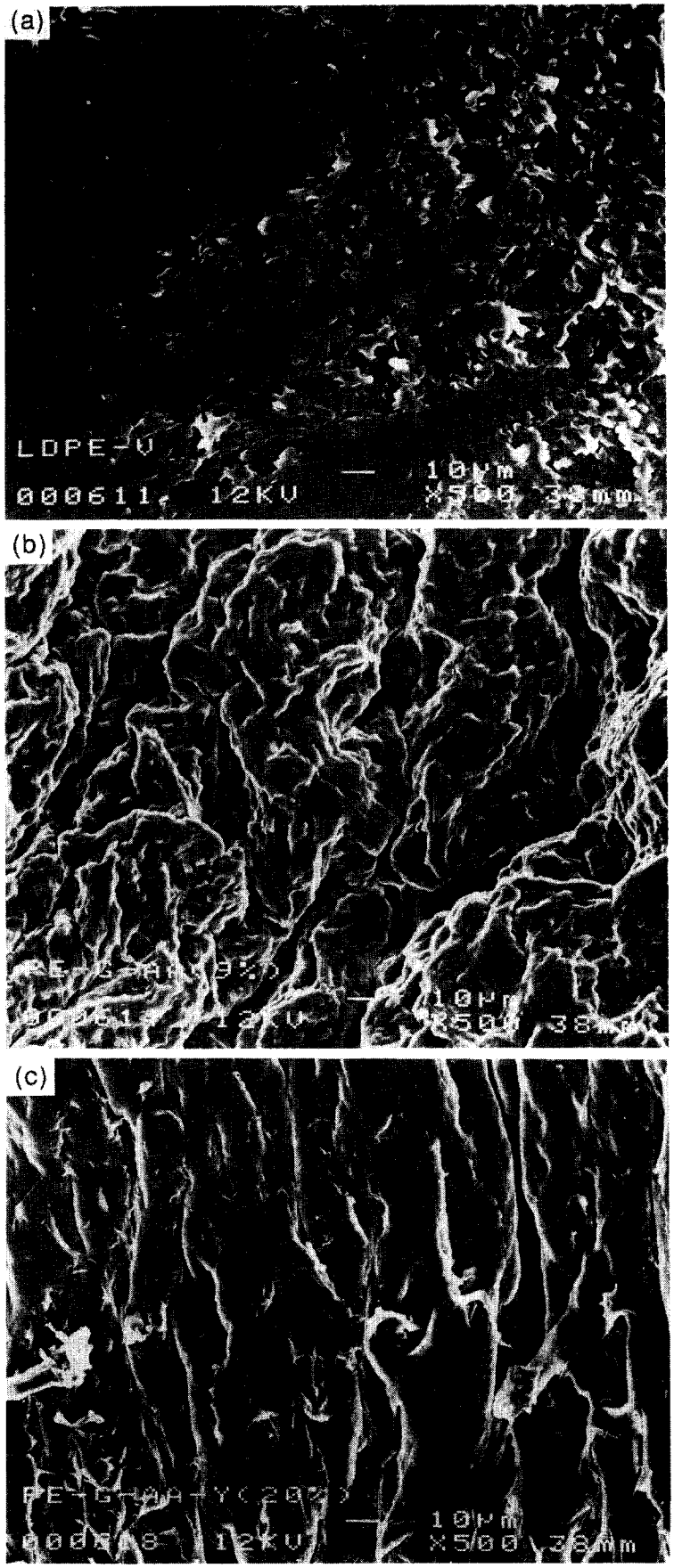

Fig. 4. Scanning electron micrographs of (a) untreated LDPE, (b) LDPE-g-AA (G: 9% (c) LDPE-g-AA (G: 20%). G (%): graft level.

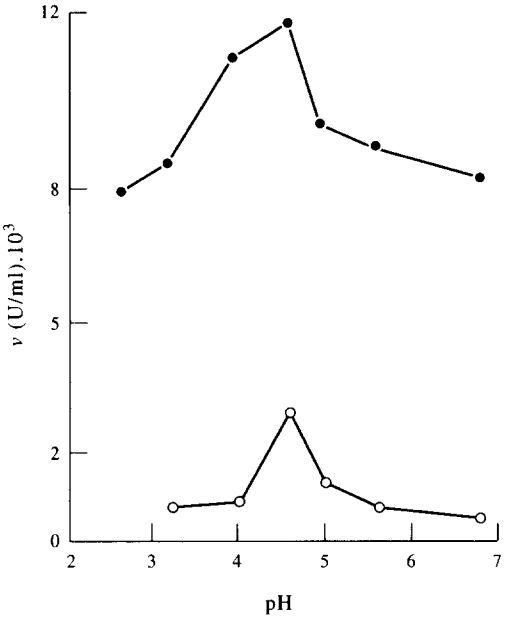

Fig. 5. Effect of pH on the activity of invertase. Activities: (●) free invertase; (○) immobilized invertase.

1987). Thus, the immobilization procedure probably maintains the oligomeric forms (mainly, octameric and hexameric aggregate) prevailing in the free invertase solution (Reddy *et al.*, 1990). The denaturation

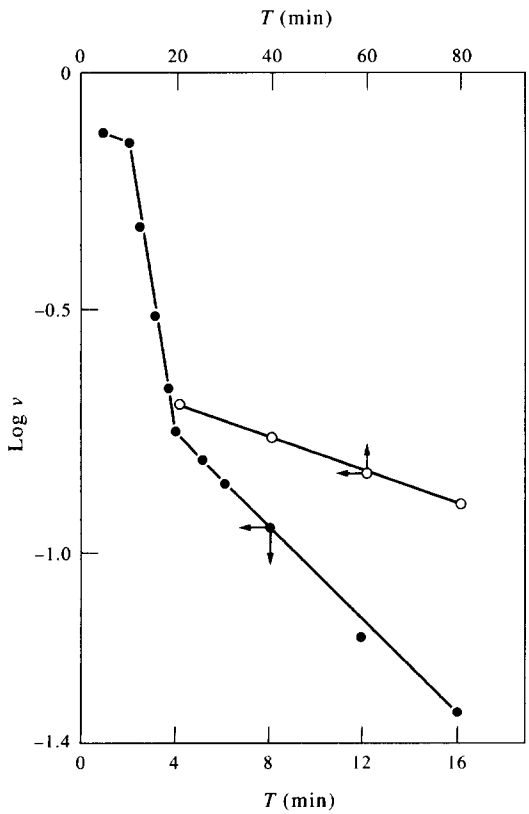

Fig. 6. Effect of temperature on the activity of invertase (●) free invertase and (○) immobilized invertase. Temperature: 70°C, pH 4.6. The arrows indicate the coordinates for each invertase forms.

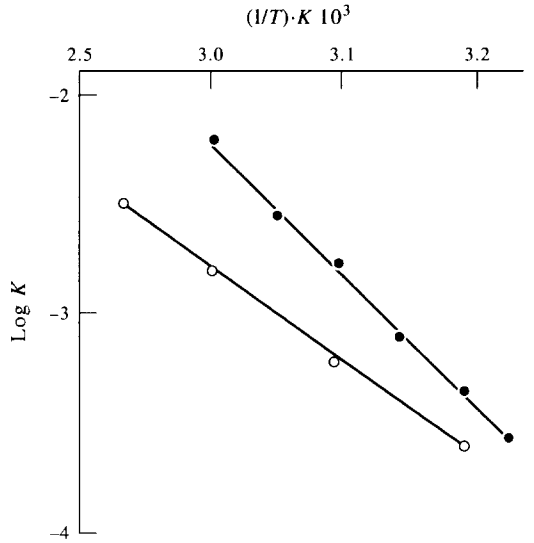

Fig. 7. Arrhenius plot for the heat inactivation of free (●) and (○) immobilized invertase.

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.

The data in Fig. 7 show that the first order inactivation rate constants (k) decreased after immobilization. This increases the potential of the immobilized invertase to be used as a practical catalyst for the inverted syrup production on an industrial scale, since higher temperatures reduce the viscosity of the sucrose solution (De-Almeida Cunha and Vitolo, 1984). This reduces the cost of the mechanical energy in agitation and other industrial operations.

Figure 7 shows the data on heat inactivation of the enzyme. The calculated thermal inactivation energy by the application of the conventional Arrhenius method for the free and immobilized enzyme was 112.5 and 79.0 kJ/mol, respectively. The other thermodynamic parameters for the heat inactivation of the free and immobilized invertase at 303–343 K are summarized in Table 2. The principal difference is in the ΔH value. The decrease of the ΔH value for immobilized invertase probably shows a higher alteration of its tertiary structure of the insoluble enzyme (Owusu *et al.*, 1992).

The straight lines of the Lineweaver–Burk plot (Fig. 8) give the Michaelis constants (K_m) as 26.5 mmol/dm^3 for the free invertase and 31.7 mmol/dm^3 for the immobilized enzyme. The increase in K_m may be due to the steric hindrance of the catalytic site of the invertase (Lantigue, 1975).

Table 2. Thermodynamic parameters related to the heat inactivation of the free and immobilized invertase

ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (kJ/mol.K)
−85.7†/−88.3‡	109.83†/76.29‡	0.609†/0.502‡

†Free invertase.
‡Immobilized invertase.

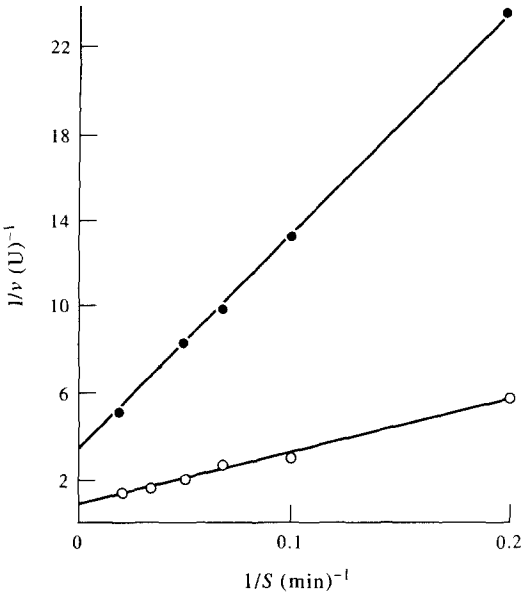

Fig. 8. Lineweaver-Burk plot for free (○) and immobilized invertase (●). The correspondent equations are: (○) free enzyme: $(1/v) = 0.914 + 23.767 \times (1/S)$ $(r = 0.9995)$ (●) immobilized enzyme: $(1/v) = 3.18 + 100.56 \times (1/S)$ $(r = 0.9990)$.

CONCLUSIONS

The synthesized acrylic enzyme carrier PAA-grafted LDPE appears to be a promising invertase carrier. The Michaelis constant, K_m, values were larger for the immobilized invertase than for the free enzyme, whereas the V_{max} values were smaller for the immobilized invertase. The optimum pH value of invertase was not affected by the immobilization reaction. The immobilized enzyme demonstrated a reduced sensitivity to thermal inactivation as compared to that of the free form.

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