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Immobilization of glucoamylase onto spacer-arm attached magnetic poly(methylmethacrylate) microspheres: characterization and application to a continuous flow reactor

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Abstract

Magnetic poly(methylmethacrylate) microspheres (MPMMA) were prepared by the solvent evaporation method and a 6-carbon spacer-arm (i.e. hexamethylene diamine, HMDA) was covalently attached by the reaction of carbonyl groups of poly(methylmethacrylate). Glucoamylase was then covalently immobilized through the spacer-arm of the MPMMA microspheres by using either carbodiimide (CDI) or cyanogen bromide (CNBr) as a coupling agent. The activity yield of the immobilized glucoamylase was 57% for CDI coupling and 73% for CNBr coupling. Kinetic parameters were determined for both immobilized glucoamylase preparations as well as for the free enzyme. The K_m values for immobilized glucoamylases CDI coupling (12.5 g l^{-1} dextrin) and CNBr coupling (9.3 g l^{-1} dextrin) were higher than that of the free enzyme (2.1 g l^{-1} dextrin) whereas V_{max} values were smaller for the immobilized glucoamylase preparations. The optimum operational temperature was 5°C higher for both immobilized preparations than that of the free enzyme. Operational, thermal and storage stabilities were found to be increased with immobilization.

Keywords: Glucoamylase; Enzyme immobilization; Covalent bonding; Spacer-arm; Enzyme reactor; Magnetic microspheres; Poly(methylmethacrylate)

1. Introduction

Immobilization of enzymes onto insoluble polymeric matrices is very a effective way to stabilize them [1,2]. Immobilization leads to heterogeneous systems which enable separation from the reaction media [3,4]. Magnetic fields have been utilized in the support systems for the study of enzyme immobilization. Rotational and vibrational movements were observed in the alternating magnetic field by polymeric beads exhibiting magnetically anisotropic properties [5–8]. In a packed bed reactor, these magnetic phenomena could be used for preventing product flim formation around the enzyme-magnetic microspheres by an alternating magnetic field. In such a system, the vibrating reaction medium around the support could facilitate substrate transfer through the surface of the enzyme-microspheres and this fact could also provide a key for controlling immobilized enzyme activity in a bioreactor.

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Amylases find a great deal of use in food and fermentation industries especially in the production of fermentable sugar. Glucoamylases attack α-1,4-linkages at the non-reducing ends of dextrin and when this reaches a α-1,6-linkage it cleaves that too but at a slower rate than the α-1,4-linkage [2,9]. Amylases have not been immobilized as frequently as the other enzymes since they act on a macromolecular substrate (i.e. starch or dextrin) and immobilization significantly reduces the reaction rate due to the low diffusion coefficient of the substrate [10,11]. Immobilizations of enzymes on the surface of support materials have been proposed to decrease mass transfer limitations.

Support materials which play an important role in the utility of an immobilized enzyme should be non-toxic, and should provide a large surface area suitable for enzyme reactions, and substrate and product transport with the least diffusional restriction. Polymeric microspheres have attracted much attention because they can be produced easily in a wide variety of compositions, and can be modified for the immobilization systems by introducing a variety of activation methods [12–14]. Poly(methylmethacrylate) (PMMA), due to its being a biocompatible synthetic polymer with adequate mechanical strength for most biomedical and biotechnological applications is regarded as a suitable matrix for immobilization of enzymes [15].

Immobilization of enzymes through covalent attachment has also been demonstrated to induce higher resistance to temperature, denaturants, and organic solvents in several cases [1–3,16–19]. The extent of these improvements may depend on other conditions of the system, i.e. the nature of the enzyme, the type of support, and the method of immobilization.

In this study, glucoamylase was covalently immobilized onto new magnetic microspheres based on poly(methylmethacrylate). For the covalent immobilization of glucoamylase onto the spacer-arm attached magnetic PMMA microspheres two different binding methods by coupling a spacer-arm either with carbodiimide or cyanogen bromide were employed. The effect of the immobilization process on the enzyme activity, the kinetic parameters, thermal and storage stabilities of the enzyme was investigated. Finally, the application of immobilized glucoamylases in an enzyme reactor is presented.

2. Experimental

2.1. Materials

Glucoamylase (amyloglucosidase (AGD), exo-1,4-α-D-glucosidase, EC 3.2.1.3 from *Aspergillus niger* 94.5 U mg^{-1}), glucose oxidase (GOD, EC 1.1.3.4. Type II from *Aspergillus niger*), peroxidase (POD, EC 1.11.1.7. Type II from horseradish), bovine serum albumin (BSA), carbodiimide (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride), *o*-dianisidine dihydrochloride, soluble dextrin, glucose, magnetite (Fe_3O_4; diameter was $< 5\,\mu m$) and sodium dodecyl sulphate were all obtained from the Sigma Chemical Company (St. Louis, USA) and used as received.

Methylmethacrylate (MMA) was obtained from Fluka AG (Switzerland), Inhibitors were removed by alkaline salt extraction (20%NaCl and 5%NaOH) washed twice with distilled water, dried with $CaCl_2$, and stored at 4°C until use. Benzoyl peroxide (BPO) hexamethylene diamine ($(NH_2)_2C_6H_{10}$; HMDA) and polyvinyl alcohol (PVA, MW 9000) were obtained from Fluka AG and used as received. All other chemicals were of analytical grade and were purchased from Merck AG (Darmstadt, Germany).

2.2. Preparation of PMMA microspheres

The PMMA microspheres were prepared by suspension polymerization. The dispersion medium consisted of tricalciumphosphate (6.0 g) in distilled water (480 ml). The discontinuous phase contained MMA (240 ml) and polymerization initiator BPO (6.0 g). The reaction was carried out at 80°C under a nitrogen atmosphere for 2 h and then for 2 h at 95°C. After the reaction, the microspheres were filtered, washed with 0.05 M HCl solution and with distilled water. The microspheres were then dried in a vacuum oven at 40°C for 24 h.

2.3. Preparation of magnetic PMMA microspheres

The magnetic poly(methylmethacrylate) microspheres were prepared by a modified solvent evaporation method [15]. The aqueous continuous phase was comprised of distilled water (400 ml) and stabi-

lizers (i.e. polyvinyl alcohol (4.0 g), sodium dodecyl sulfate (2.0 g), and Pluronic F6800 (6% (v/v), 5 ml)). The PMMA microspheres (20.0 g) were transferred in a closed bottle containing chloroform (100 ml) and stirred magnetically at 400 rpm for 18 h at room temperature. The magnetite (0.5 g) was then added to this polymer solution and mixed uniformly. This mixture was transferred into a glass reactor (1000 ml) containing aqueous continuous phase (400 ml) and stirred at 700 rpm with a mechanical stirrer for 18 h at 20°C. After solvent evaporation period, the magnetite particles were completely encapsulated in the formed PMMA microspheres. The magnetic microspheres were washed several times with distilled water and dried in a vacuum oven at 45°C. Tyler series sieves were used to separate the microspheres have the diameter between 100 and 200 μm.

2.4. Spacer-arm attachment onto the magnetic PMMA microspheres

The covalent attachment of the 6-carbon spacer-arm (HMDA) onto the magnetic PMMA microspheres was carried out by the following method. The magnetic PMMA microspheres (10.0 g) were transferred into tetrahydrofuran (60 ml) containing HMDA (10.0 g) and NaH (3.0 g). The reaction medium was boiled at 67°C in a reactor and refluxed for 20 h. After this period, the medium was cooled down to room temperature and methanol was added dropwise until the end of gas formation. The HMDA derived magnetic PMMA microspheres were washed and then dried in a vacuum oven. They were stored at 4°C until use.

2.5. Immobilization of glucoamylase onto magnetic PMMA microspheres

Two different coupling methods were utilized for the immobilization of glucoamylase on the spacer-arm attached magnetic PMMA microspheres. In the first method, carbodiimide was used as a coupling agent for the immobilization of glucoamylase through carboxylic groups. The magnetic microspheres (10 g; diameter 100–200 μm) were equilibrated in phosphate buffer (50 mM, pH 7.5) for 2 h. It was then transferred to the same fresh buffer (40 ml) containing amyloglocosidase (2 mg ml^{-1}) and carbodiimide (10 mg). The reaction was carried out at 4°C for 18 h while continuously stirring the medium. The enzyme-immobilized microspheres were washed with NaCl solution (20 ml, 0.5 M) and then with phosphate buffer (50 mM, pH 7.0). In the second method, CNBr was employed for the coupling of enzyme on the spacer-arm attached magnetic PMMA microspheres. An aqueous solution of CNBr (20 ml, 2 mg ml^{-1}) was prepared in distilled water and the solution pH was adjusted to 11.5 with 0.2 M NaOH. The magnetic microspheres (10.0 g) were transferred in this medium and stirred magnetically at 25°C for 60 min. After the reaction period, the activated microspheres were collected and the remaining CNBr was removed by washing first with 0.1 M $NaHCO_3$, and then distilled water. The immobilization of glucoamylase onto CNBr activated microspheres was carried out in carbonate buffer (0.1 M, pH 9.5, containing 2 mg ml^{-1} glucoamylase) at 4°C for 18 h. After the coupling reaction, the enzyme-microspheres were washed as described above.

2.6. Determination of immobilization efficiency

The amount of protein in the enzyme solution and in the wash solutions was determined by using Coomassie Brilliant Blue as described by Bradford [20] with BSA as a standard. The amount bound enzyme was calculated as

$$q = \frac{[(C_i - C_f)V]}{W} \quad (1)$$

where q is the amount of bound enzyme onto magnetic PMMA microspheres (mg g^{-1}), C_i and C_f the concentrations of the enzyme initial and final in the reaction medium, respectively (mg ml^{-1}), V the volume of the reaction medium (ml), W the weight of the microspheres (g). All data used in this formula are averages of at least duplicated experiments.

2.7. Activity measurements

The activities of both the free and the immobilized glucoamylase preparations were determined by measuring the glucose content in the medium according to a method described previously [2]. Assay mixture contained GOD (25 mg), POD (6.0 mg) and *o*-dianisidine (13.2 mg) in phosphate buffer (100 ml,

0.1 M, pH 7.0). An aliquot (2.5 ml) and 0.1 ml of enzymatically hydrolysed sample were mixed and then incubated at 35°C for 30 min in a water bath. After addition of sulfuric acid (1.5 ml, 30%) absorbance was measured at 525 nm in a UV/VIS spectrophotometer (Shimadzu, Model 1601; Tokyo, Japan).

Kinetic parameters (K_m and V_{max} values) of the free enzyme were determined by measuring initial rates of the reaction with soluble dextrin (1.0–20.0 g l^{-1}) in acetate buffer (50 mM, pH 5.5) at 50°C. One unit of glucoamylase activity is defined as the amount of enzyme which produces 1.0 mmol glucose from dextrin in 1.0 min at 50°C at pH 5.5.

2.8. Effect of temperature and pH on free and immobilized glucoamylase activity

The effect of temperature on enzyme productivity was studied in the range 20–60°C in a batch operation with a soluble dextrin concentration of 10.0 g l^{-1} in acetate buffer (50 mM, pH 5.5). The effect of pH on the activity of free and immobilized enzyme was investigated at 50°C. The concentration of the soluble dextrin was 10.0 g l^{-1} and was prepared in acetate buffer (50 mM) in the pH range 4.0–5.5 and in phosphate buffer (50 mM) in the range pH 6.0–8.0.

2.9. Continuous use of immobilized glucoamylases in a packed bed reactor

The reactor (length 12.0 cm, radius 0.6 cm, total volume 13.6 ml), was made from Pyrex^R glass. The reactor was charged with immobilized glucoamylase (weight of enzyme-magnetic microspheres 10.0 g) yielding a void volume of about 4 ml. In order to determine operational stability of immobilized glucoamylase, the reactor was operated at 55°C for 60 h with soluble dextrin (10.0 g l^{-1} in the acetate buffer (50 mM, pH 5.5) which was introduced to the reactor at a flow rate of 80 ml h^{-1} (the residence time was 3 min) with a peristaltic pump (Cole Parmer, Model 7521-00,USA) through the lower inlet part. The solution leaving the reactor was collected by means of a fraction collector. At the end of each hour, the collected sample was assayed for immobilized glucoamylase activity according to the method given in the activity assay section.

2.10. Thermal and storage stability measurements of free and immobilized enzymes

The thermal stability of the free and immobilized glucoamylase preparations were determined by measuring the residual activity of the enzyme exposed to two different temperatures (60 and 70°C) in acetate buffer (50 mM, pH 5.5) for 4 h. A sample was removed at each 0.5 h and assayed for enzymatic activity. The first-order inactivation rate constant, k_i was calculated from the equation

$$\ln A = \ln A_0 - k_i t \quad (2)$$

where A_0 and A are the initial activity and the activity after time t (min).

The activity of free and both immobilized glucoamylase preparations after storage in acetate buffer (50 mM, pH 5.5) at 4°C was measured in a batch operation mode with the experimental conditions as given above.

2.11. Characterisation of magnetic PMMA microspheres

2.11.1. Analysis of magnetism

The degree of magnetism of the microspheres were measured in a magnetic field by using a vibrating-sample magnetometer (Princeton Applied Research Corporation, USA). The presence of magnetite in the polymeric structure was investigated with ESR spectrophotometer (Model El 9, Varian).

2.11.2. Scanning electron microscopy

Scanning electron micrographs of the magnetic microspheres were obtained using a Leitz AMR-1000 (Germany) after coating with gold under vacuum.

2.11.3. Elemental analysis

The amount of attached hexamethylene diamine onto magnetic microspheres was determined from elemental analysis device (Leco, CHNS-932, USA).

2.11.4. FTIR spectra

The FTIR spectra of the plain and hexamethylene diamine attached magnetic microspheres were obtained by using a FTIR spectrophotometer (Shimadzu, FTIR 8000 Series, Japan). The microspheres (0.1 g) and KBr (0.1 g) were thoroughly

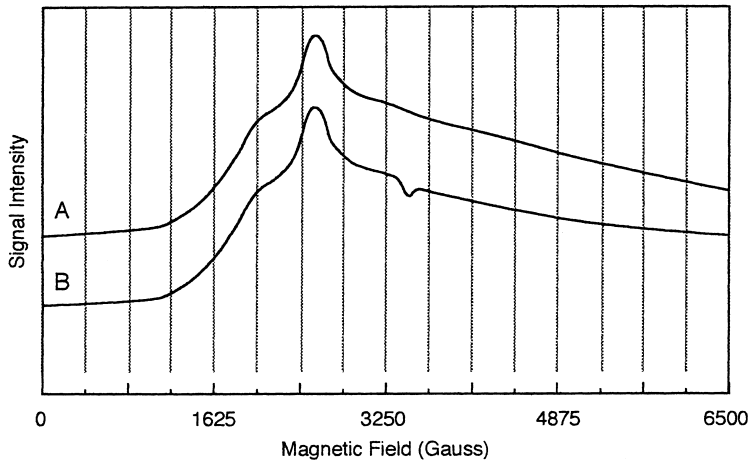

Fig. 1. The ESR Spectra of magnetic PMM microspheres; (A) unmodified microspheres; (B) HMDA attached microspheres.

mixed and this mixture was pressed to form a tablet, and the spectrum was recorded.

3. Results and discussion

3.1. Properties of magnetic PMMA microspheres

The presence of magnetic in the PMMA structure was confirmed by electron spin resonance (ESR). A peak of magnetite (i.e. Fe_3O_4 fine particle) was detected in the ESR spectrum and is presented in Fig. 1. It was observed that in the same magnetic field region, the unmodified and the hexamethylene diamine attached PMMA microspheres gave similar magnetic peak intensities. The behavior of magnetic microspheres in a magnetic field was determined using a vibrating magnetometer, and the results are presented in Fig. 2. The electromagnetic unit (emu)

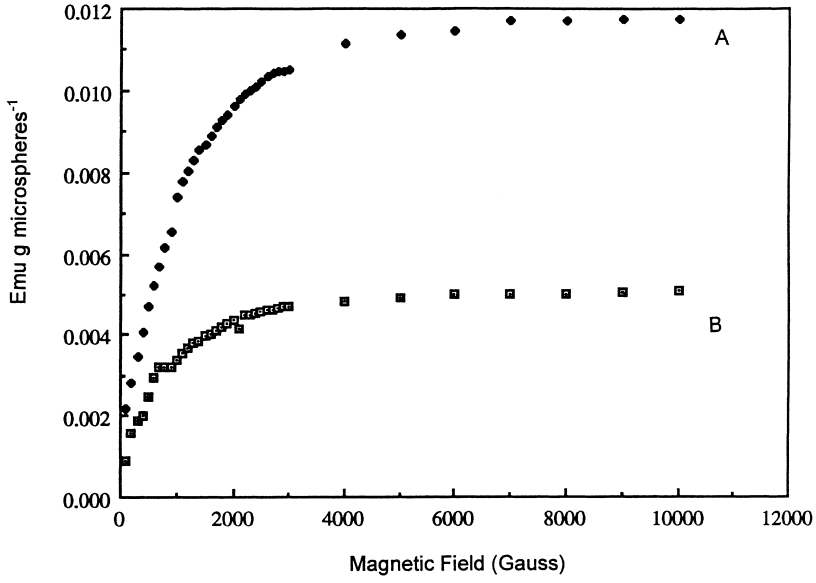

Fig. 2. The magnetic behavior of PMMA microspheres; (A) unmodified microspheres; (B) HMDA attached microspheres.

is related to the intensity of magnetization of the sample versus applied magnetic field. In these spectra, 3000 G magnetic field was found sufficient to excite all of the dipole moments of 1.0 g of microspheres sample that consists of magnetite. After the hexamethylene diamine attachment on the magnetic microspheres, the intensity of magnetization of the sample is affected with the applied magnetic field. Magnetic field intensity is a very important design parameter for a magnetically stabilized reactors with immobilized enzyme systems for the magnetic filtration systems. Bahar and Çelebi [21] reported that by applying different magnetic field intensities, the void volume of the fluidized bed reactor could be adjusted, and the pressure drop in the system could be controlled. The value of this magnetic field is a function of the flow velocity, particle size and magnetic susceptibility of solids to be displaced. In the literature, this value changes from 8000 to 20,000 G for various applications, thus our magnetic microspheres will need less magnetic intensity in a magnetic bed or a magnetic filter.

Fig. 3 shows the SEM micrograph of the magnetic microspheres. As clearly seen, the microspheres have a rough surface due to the abrasion of magnetic crystals (diameter < 5 mm) during the coating procedure. These surface properties of the magnetic microspheres would favor higher immobilization capacity for the enzyme due to increase in the surface area.

Ester groups in the PMMA structure were converted to amino groups by reacting with the hexmethylene diamine as a 6-carbon spacer-arm. Elemental analysis of the HMDA attached magnetic microspheres were performed, and the amounts of the incorporated amino group were found to be 165 mmol g^{-1} magnetic microspheres from the nitrogen stoichiometry.

The FTIR spectra of unmodified and HMDA-attached magnetic microspheres are presented in Fig. 4. The FTIR spectra of HMDA-attached magnetic microspheres have some absorption bands different to those of the unmodified magnetic microspheres. The most important adsorption bands at 1665 and 1550 cm^{-1} represent amide I (C=O stretching) and amide II (N–H bending), respectively, and are due to HMDA bonded to the magnetic microspheres.

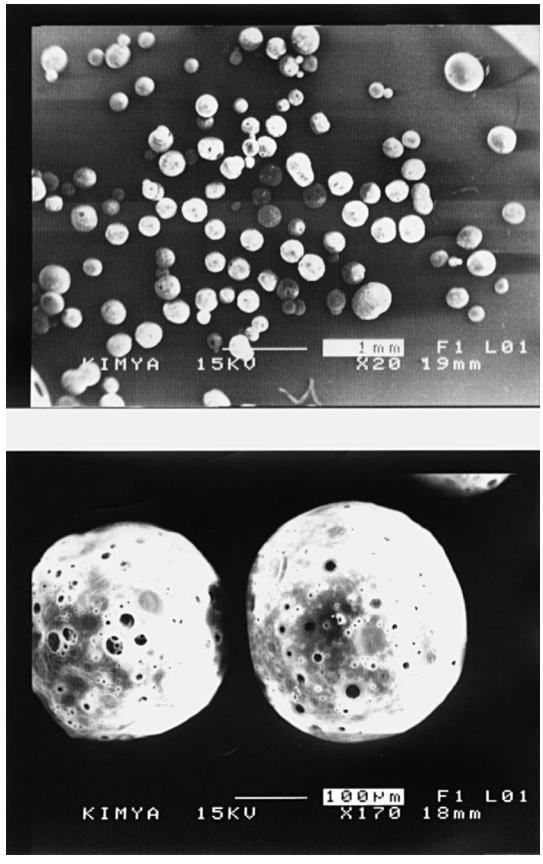

Fig. 3. The SEM micrograph of magnetic PMM microspheres.

3.2. Immobilization of glucoamylase onto magnetic PMMA microspheres

Two different coupling methods were used for the covalent immobilization of glucoamylase on the HMDA attached microspheres. In the first method, the HMDA attached magnetic PMMA microspheres and condensing agent (in this case carbodiimide) are added simultaneously to the enzyme solution. The amino or hydrazine group-containing supports provide a method of binding enzyme via their carboxyl groups. During the condensation reaction amide bonds are formed between amino groups of the support and carboxyl groups of the enzyme (Fig. 5). In the second method, the activation of amino groups of the HMDA attached magnetic microspheres was achieved by the reaction with cyanogen bromide

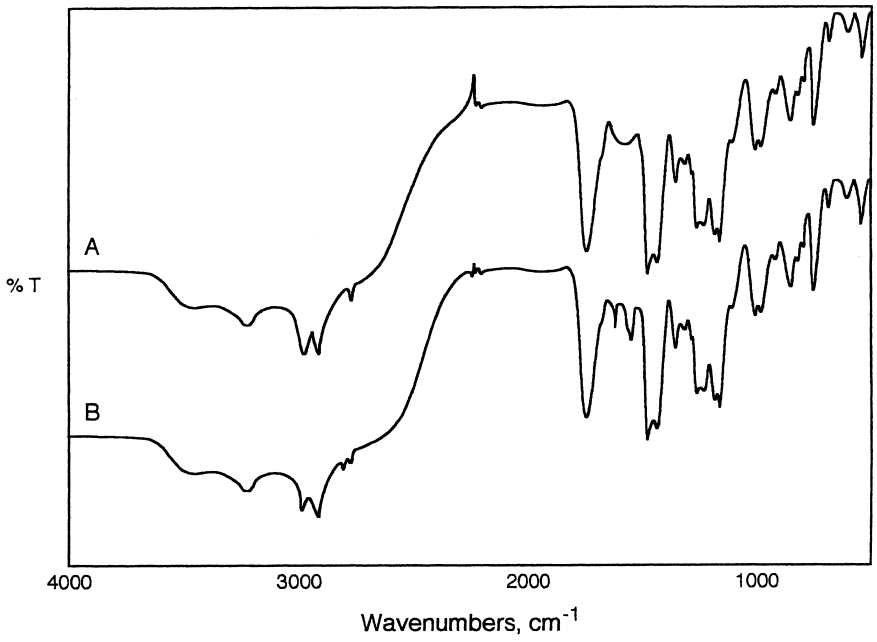

Fig. 4. The FTIR Spectra of magnetic PMM microspheres; (A) unmodified microspheres; (B) HMDA attached microspheres.

under alkaline conditions. Glucoamylase was then covalently immobilized via amino group to the activated magnetic support. The amino group of the enzyme undergoes to the formation of a guanidino linkage between the enzyme and support (Fig. 5).

A spacer-arm comprising aliphatic chains of 6-carbon atoms has been used to separate-immobilized glucoamylase from the support. The attachment of 6-carbon atom hydrophobic spacer-arms on the magnetic microspheres surface could prevent undesirable side interactions between the large enzyme molecule and the support. In this way, all areas of the immobilized glucoamylase could become fully accessible to its large substrate soluble dextrin.

The amount of enzyme which was bound onto HMDA attached magnetic microspheres with these two immobilization methods and their resultant activities are presented in Table 1. The concentration of the soluble dextrin in the activity assay medium was $20\,g\,l^{-1}$. The conditions were the same for the free and the immobilized preparations as described for the activity assays. As seen in Table 1, the immobilization of glucoamylase through amino groups via cyanogen bromide coupling onto magnetic microspheres resulted in a higher protein loading (4.35 mg protein g/microspheres) and a higher activity yield ($262.4\,U\,g^{-1}$ microspheres) than the immobilization of protein through carboxyl groups via carbodiimide coupling, (2.56 mg protein g/microspheres) and ($120.5\,U\,g^{-1}$ support), respectively. The relative activity is the effective activity after immobilization referred to the activity which the bound protein amount would have in solution. A low relative activity indicates that much of the protein was apparently immobilized in a nonactive form. As seen in Table 1 the relative activity was higher (ca. 73%) when glucoamylase immobilized via CNBr coupling instead of via CDI coupling (ca. 57%).

3.3. Kinetic constants

Kinetic parameters, the Michaelis constant K_m and the V_{max} for free and immobilized glucoamylases were determined using soluble dextrin as substrates (Table 1). In the free and immobilized enzyme preparations, Michaelis–Menten kinetic behavior was observed. The Lineweaver–Burk plots for the free and immobilized glucoamylases are presented in Fig. 6; K_m and V_{max} values were

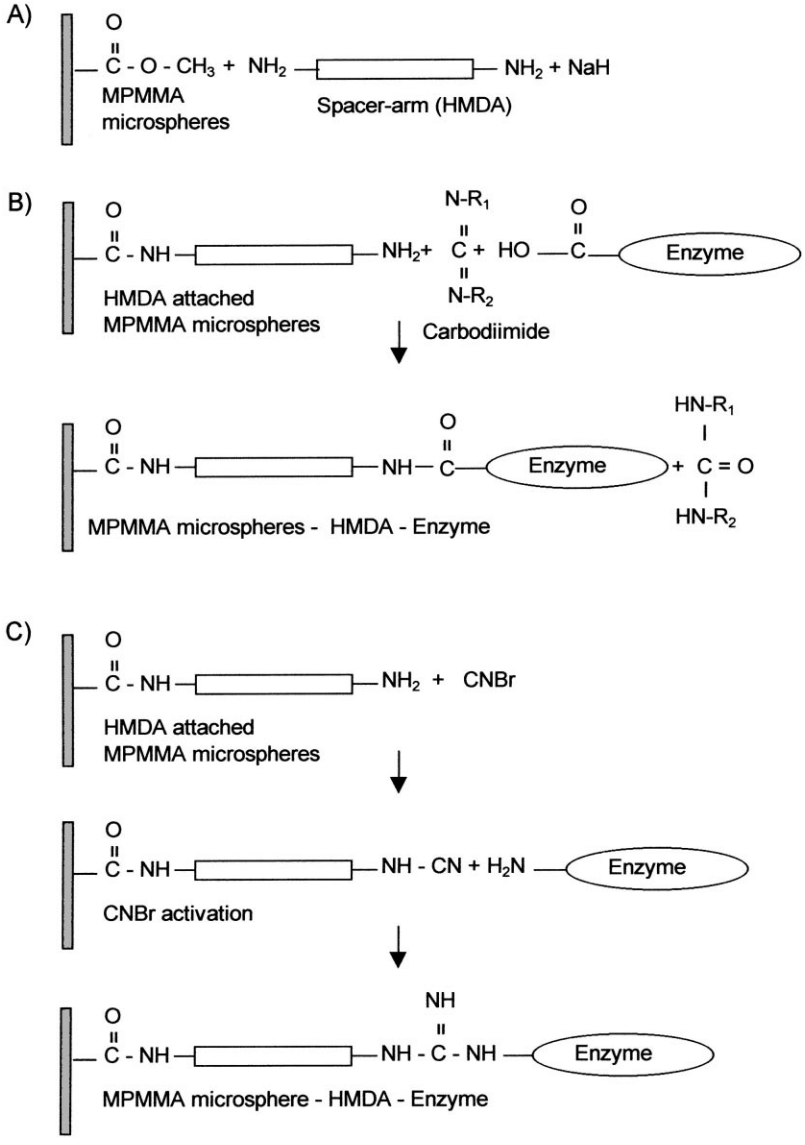

Fig. 5. Schematic representation of reaction mechanisms; (A) spacer-arm attachment; (B) immobilization of enzyme via CDI coupling; (C) immobilization of enzyme via CNBr coupling.

calculated from the intercepts on *x*- and *y*-axes, respectively. For the free enzyme, K_m was found to be $2.1\,g\,l^{-1}$ whereas V_{max} was calculated as $82.7\,U\,mg^{-1}$ of enzyme). Kinetic constants of the immobilized glucoamylase were also determined in the batch system. The K_m values were found to be $12.5\,g\,l^{-1}$ for CDI coupled enzyme and $9.3\,g\,l^{-1}$ for CNBr coupled enzyme. The V_{max} values of immobilized enzyme for CDI and CNBr coupled preparations were estimated from the data as 47.1 and $60.4\,U\,mg^{-1}$ of immobilized enzyme onto the magnetic microspheres, respectively**.** Bahar and Çelebi immobilized glucoamylase on magnetic polystyrene particles, the K_m value of the immobilized glucoamylase was three times higher than that of the free enzyme, and the V_{max} of the immobilized glu-

Table 1
Enzyme loading and the kinetic properties of the free and the immobilized glucoamylases[a]

Glucoamylase	K_m (g dextrin l^{-1})	V_{max} (U mg^{-1} enzyme)	Bound protein (mg g/support)	U g^{-1} support[b]	Relative activity (%)
Free	2.1	82.7	–	–	100
Immobilized viaCDI Coupling	12.5	47.1	2.56	120.5	57
Immobilized viaCNBr Coupling	9.3	60.4	4.35	262.4	73

[a] A standard curve was prepared with glucose solutions of different concentration and the slope of the curve was used in the quantification of glucose in the sample.
[b] U g/support = (μmol glucose in ml enzymatically hydrolysed mixture per minute)/(g microspheres).

coamylase was about 70% of the free enzyme [21]. In our study, as expected, the K_m and V_{max} values were significantly affected after covalent immobilization of glucoamylase onto magnetic microspheres. These effects were more pronounced when CDI was used as a coupling agent. The change in the affinity of the enzyme to its substrate is probably caused by structural changes in the enzyme introduced by the immobilization procedure and by lower accessibility of the large substrate to the active site of the immobilized enzyme [2].

3.4. Effect of pH on activity

The effect of pH on the activity of free and immobilized glucoamylase in dextrin hydrolysis was determined in the pH range 4.0–8.0 and the results are presented in Fig. 7. The maximum activity for free enzyme was observed at pH 5.5. The optimum pH value of free enzyme shifted 0.5 unit to the acidic and alkaline region after immobilization of glucoamylase via CNBr coupling and CDI coupling on the HMDA attached magnetic microspheres, respectively. It is known that polyionic matrices cause the partitioning of protons between the bulk phase and the enzyme microenvironment causing a shift in the optimum pH value. The shift depends on the method of immobilization as well as on the structure and charge of the matrix [14,22,23].

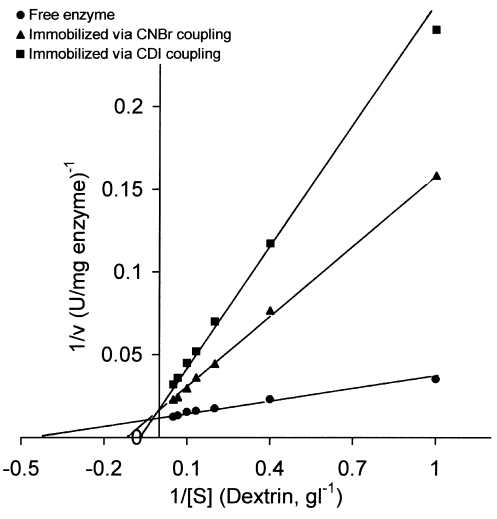

Fig. 6. Lineweaver–Burk plot for the free and the immobilized glucoamylases.

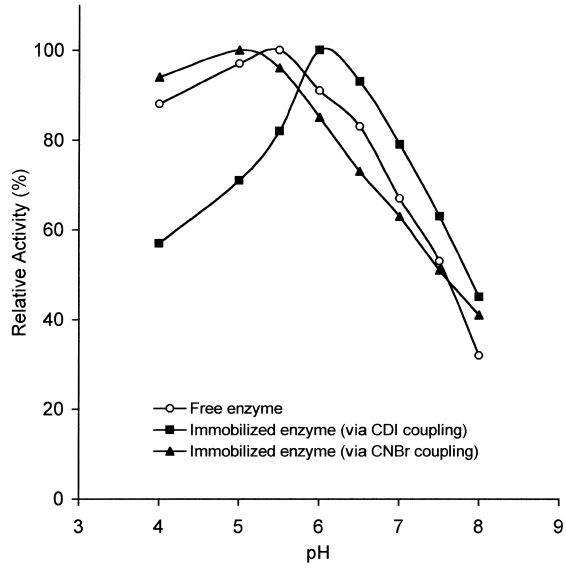

Fig. 7. The pH profiles of the free and the immobilized glucoamylases.

3.5. Effect of temperature on activity

Maximum activity for free and both immobilized preparations were observed at 50 and 55°C, respectively (Fig. 8). The increase in optimum temperature was caused by the changing physical and chemical properties of the enzyme. This could be explained by creation of conformational limitations on the enzyme movements as a result of formation of covalent bonds between the enzyme and the support. In general, the effect of changes in temperature on the rates of enzyme-catalyzed reactions does not provide much information on the mechanism of biocatalysts. However, these effects can be important in indicating structural changes in enzyme [22]. Arrhenius plots in the temperature range from 20°C to optimum appear linear and activation energies were found to be 1.74, 2.38 and 2.68 kcal mol^{-1} for free and immobilized enzyme preparations (CNBr and CDI coupled), respectively. The activation energy of an enzyme reaction may or may not change as a consequence of the immobilization process. For example, the activation energies of immobilized glucoamylase [24] and β-galactosidase [25] were almost the same as their free counterparts. On the other hand, the activation energy of invertase covalently immobilized onto pHEMA membrane increased in comparison to that of the free enzyme [12].

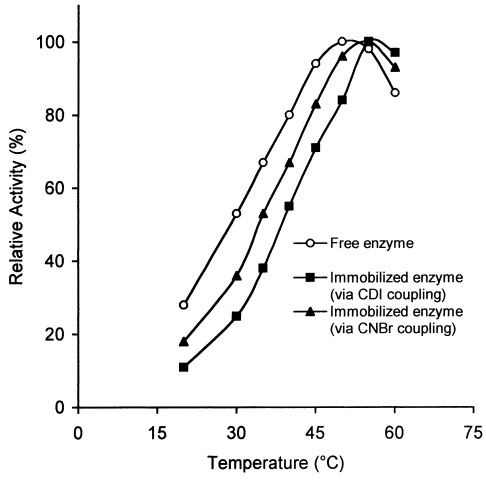

Fig. 8. Temperature profiles of the free and the immobilized glucoamylases.

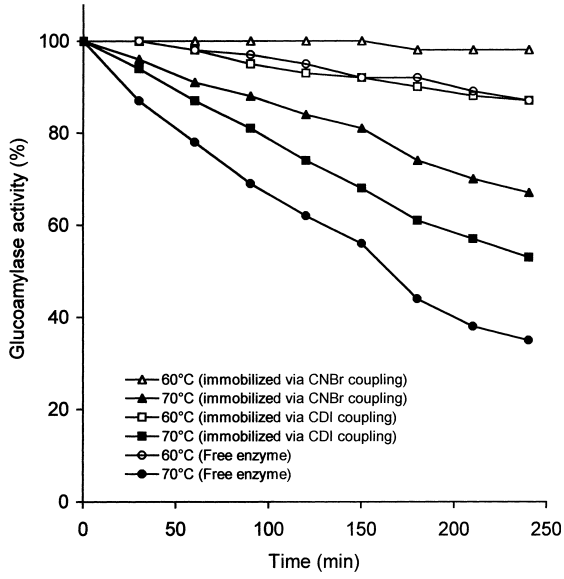

Fig. 9. Effect of temperature on the stability of the free and the immobilized glucoamylases.

3.6. Thermal stability

The effect of temperature on the thermal stability of free and both immobilized enzymes are presented in Fig. 9. The free glucoamylase retained about 87 and 35% of its initial activity at 60 and 70°C after a 240 min incubation period, respectively. After a 240 min treatment at 60°C, immobilized glucoamylases via CNBr and CDI coupling retained about 98 and 93% of their initial activities. At 70°C, these were 53 and 67%, respectively. The half-lives and the thermal inactivation rate constant at 70°C were determined and presented in Table 2. These results

Table 2
Thermal inactivation rate constant (k_i) and half-life ($t_{1/2}$) of the free and immobilized glucoamylase at 70°C

Glucoamylase	Thermal inactivation rate constant (k_i) (10^{-3} min^{-1})	Half-life ($t_{1/2}$) (min)
Free	4.36	184
Immobilized via CDI coupling	2.63	255
Immobilized via CNBr coupling	1.65	363

suggest that the thermostability of immobilized glucoamylase becomes significantly higher than that of the free enzyme at high temperature. This is due to the covalently immobilized enzyme being protected from conformational changes caused by the environment. Similar results have been previously reported for various covalently immobilized enzymes [2,4,12,22].

3.7. Operational stability

The operational stability of immobilized glucoamylases was studied in the packed bed reactor for 60 h. CNBr coupled and CDI coupled immobilized glucoamylases lost about 8% (from 262.4 to 241.4 U support g^{-1}) and 11% (from 120.5 to 107.2 U support g^{-1}) of their initial activities after 60 h of the continuous operation (Fig. 10). The operational inactivation rate constants (k_{iop}) of immobilized enzymes at 50°C with 10.0 g l^{-1} dextrin in acetate buffer (50 mM, pH 5.5) were calculated to be 2.31×10^{-5} min^{-1} for CNBr coupled and 3.12×10^{-5} min^{-1} for CDI coupled glucoamylase. Thus, the high operational stabilities obtained with immobilized glucoamylase preparations indicate that these immobilized enzyme preparations can successfully be used for continuous production of glucose from soluble dextrin.

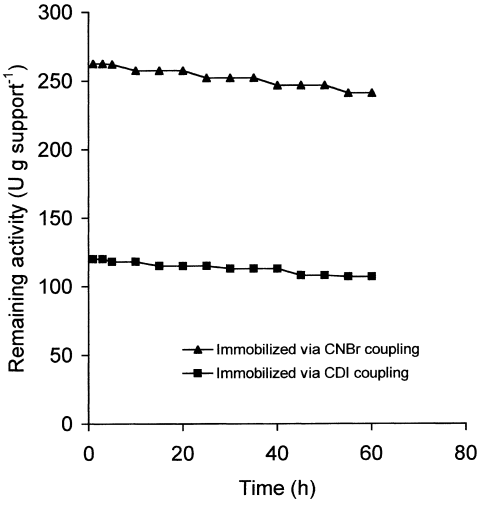

Fig. 10. Operational stability of the immobilized glucoamylases in continuous flow reactor.

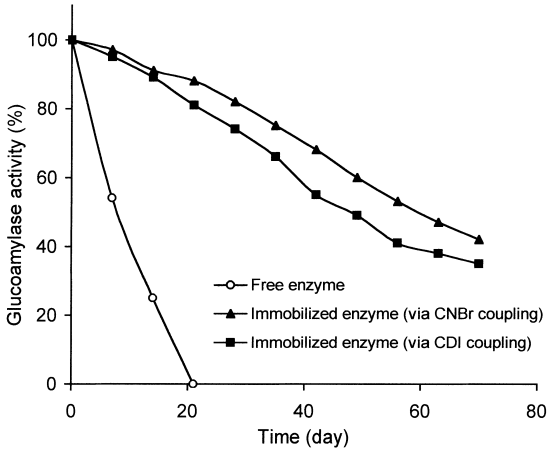

Fig. 11. Storage stability of the free and the immobilized glucoamylases.

3.8. Storage stability

Free and immobilized glucoamylase preparations were stored in acetate buffer (50 mM, pH 5.5) at 4°C and the activity measurements were carried out for a period of 70 days (Fig. 11). The free enzyme lost its all-initial activity within 21 days. Immobilized preparations (via CNBr and CDI coupling) lost 12 and 18% of their activities during the same period. As can be seen, the immobilization definitely holds the enzyme in a stable position in comparison to the free counterpart. The storage stability of immobilized glucoamylase preparations was more than 80% higher than that of the free enzyme.

4. Conclusion

The porous magnetic PMMA microspheres (diameter between 100 and 200 mm) were utilized for glucoamylase immobilization. Generally, small particles are used for enzyme immobilization because of their high immobilization area but packed-bed reactors with small particles show high pressure drops and insufficient flow rates for large scale applications. These disadvantages arising from small size could be eliminated by using of magnetic particles. Glucoamylase was immobilized using two different coupling agents (i.e. CNBr and CDI) on the mag-

netic support. Upon immobilization of glucoamylase on the magnetic microspheres, especially the CNBr coupling method yielded a high residual activity, and a high operational, thermal, and storage stability than that of the CDI coupling method. A high operational stability obtained with these preparations indicates that these immobilized enzymes can successfully be used for continuous production of glucose from soluble dextrin.

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