ELSEVIER

Food Chemistry 85 (2004) 461–466

Food Chemistry

www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Covalent immobilization of invertase on microporous pHEMA–GMA membrane

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Received 4 April 2003; received in revised form 8 July 2003; accepted 8 July 2003

Abstract

Poly (2-hydroxyethyl methacrylate–glycidyl methacrylate) (pHEMA–GMA) membrane was prepared by UV-initiated photopolymerization. Invertase was immobilized by the condensation reaction of the epoxy groups of glycidyl methacrylate in the membrane structure with amino groups of the enzyme. The K_m values were 22 mM and 58 mM for free and immobilized enzyme, respectively. Immobilization improved the pH stability and temperature stability of the enzyme. Thermal stability was found to increase with immobilization. The half times for the activity decay at 70 °C were found to be 11 and 38 min for the free and immobilized enzyme, respectively. The immobilized enzyme activity was found to be quite stable in later experiments.

Keywords: pHEMA–GMA membrane; Epoxy groups; Covalent bonding; Enzyme immobilization; Invertase

1. Introduction

Enzymes are biological catalysts that increase the rate of chemical reactions taking place within living cells by lowering the energy of activation (Erginer, Toppare, Alkan, & Bakır, 2000) They may be used in industry in free or immobilized forms. Immobilization may offer additional stability to a variety of enzymes against several denaturing forms. Enzymes have been immobilized on different supports, using methods such as entrapment, adsorption, or covalent binding (Mosbach, 1987; Sosnitza, Farooqui, Saleemuddin, Ulber, & Scheper, 1998). Enzyme immobilization can affect the stability, pH and temperature optima, Michelis–Menten constant (K_m) and maximum reaction rate (V_{max}) of an enzyme. Membrane-immobilized enzymes may serve as model systems for enzymes or may find practical application in enzyme reactors and enzyme electrodes as less expensive, more stable, and reusable alternatives to free enzymes (Arıca, Şener, Aleaddinoğlu, Patyır, & Denizli, 2000).

In this study, immobilization of invertase using poly (2-hydroxyethyl methacrylate–glycidyl methacrylate) (pHEMA–GMA) membranes as a support, has been undertaken. For a carrier to be suitable for enzyme immobilization, it must have a large surface area with a high content of the reactive group, good mechanical and chemical stability and good flow properties. According to earlier studies, pHEMA is a nontoxic, biocompatible synthetic polymer with adequate mechanical strength for most biotechnological applications. One other advantage is the presence hydroxyl groups that act as attacment sites for bioactive species after activation or introduction of different functional groups to the polymer chain (Arıca, Denizli, Salih, Piþkin, & Hasırcı, 1997; Denizli & Piþkin, 1995; Piedade, Gil, Cavaco, Andrade, 1995).

Due to these properties, pHEMA has been used in previous studies to immobilize enzymes and cells after preparation via various techniques, including suspension, bulk, and solution polymerization using thermal, chemical, and γ-irradition as initiators (Gürsel, Arıca, & Hasırcı, 1997).

Invertase, known as β-fructofuranosidase (EC 3.2.1.26), plays a catalytic role in the conversion of sucrose into glucose and fructose. A membrane-immobilized invertase can be used in the analytical field for the construction of sucrose biosensors and in enzyme reactors for hydrolysis of sucrose. The product obtained by invertase has the advantage of being colourless

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doi:10.1016/j.foodchem.2003.07.015

compared to the coloured version obtained through acid hydrolysis (Kotzelski & Staude, 1996; Mansfeld, Förster, Schellenberger, & Dautzenberg, 1991; Marek, Valentino, & Kas, 1984;). The mixture of these products has a lower crystallinity than sucrose at high concentrations and does not crystallize out like sucrose. Its use in confectionery thus ensures that the products remain fresh and soft even when kept for a long time. Therefore, it is widely used in the production of noncrystallizing creams, for making jams and is also used in the production of artificial honey, and to a small extent in the industrial production of liquid sugar (Hartmeimer, 1998).

Immobilization of invertase on natural polymers such as gelatin, corn grits or agarose, has already been achieved (Akbulut, Sungur, & Pekyardımcı, 1991; Marek et al., 1984; Monsan & Combes, 1984). Its immobilization onto hydrogel polymers is limited. Hydrogel polymers have good chemical and mechanical stability, and are not susceptible to microbial attack (Arıca, Hasırcı, & Alaeddinoğlu, 1995; Cantarella, Cantarella, & Alfani, 1993).

Differently functionalized carrier materials and various coupling methods have been used to illustrate the influence of immobilization on kinetic behaviour of membrane-bound invertase. In the present study, the aim was to immobilize invertase with a significantly higher activity and stability than those described in earlier studies. Invertase was covalently immobilized onto an epoxy group pHEMA membrane. The resultant immobilized invertase system was characterized and its activity retention, catalytic properties, reusability, and stability aspects were compared.

2. Materials and methods

2.1. Materials

Invertase (β-fructofuranosidase, EC 3.2.1.26, Grade VII from baker's yeast), glucose oxidase (GOD, EC 1.1.3.4, Type II from *Aspergillus niger*), peroxidase (POD, EC 1.11.1.1. Type II from horseradish), bovine serum albumin (BSA), *o*-dianisidine dihidrochloride, sucrose and glucose were all obtained from the Sigma Chemical Company (St. Louis, USA) and used as received.

2-Hydroxyethyl methacrylate (pHEMA), glycidyl methacrylate (2,3-epoxypropyl methacrylate) (GMA), and α,α′-azoisobutyronitrile (AIBN) were obtained from Fluka AG (Switzerland) and stored at 4 °C until used. All other chemicals were of analytical grade and were purchased from Merck AG (Darmstadt, Germany).

2.2. Membrane preparation

The pHEMA membrane was prepared as previously described (Aryıca, & Hasırcı, 1987). The membrane preparation mixture (5 ml) contained 2 ml (HEMA), 0,75 ml (GMA) and 5 mg AIBN as polymerization initiator and 2.25 ml phosphate buffer (pH 7.0, 0.1 mol dm^{-3}). The mixture was then poured into a round glass mould (diameter 9 cm) and exposed to ultraviolet radiation (12 W lamp) for 15 min, while a nitrogen atmosphere was maintained in the mold. The membrane was washed several times with distilled water and cut into circular pieces (diameter 1.0 cm) using a perforator.

2.3. Immobilization of invertase on membranes through covalent bonding

The epoxide-containing membrane was swollen in phosphate buffer (50 mM) for 2 h, immersed in enzyme solution (1 mg ml^{-1}). The immobilization of invertase was carried out at 4 °C for 24 h, while continuously stirring the enzyme solution. The GMA-derived pHEMA membrane containing invertase was washed several times with acetate buffer (0.1 M, pH 5.5)

2.4. Protein determination

The measurements of protein in the enzyme solution and in the wash solutions were performed by the Bradford method (1976). BSA solution (1 mg ml^{-1}) was used as a standard.

2.5. Activity assays

The activities of both the free and the immobilized invertase preparations were determined by measuring the amount of glucose liberated from the invertase-catalyzed hydrolysis of sucrose per unit time.

In the determination of the activity of the free enzyme,the reaction medium consisted of acetate buffer (2.5 ml, 50 mM, pH 5.0), sucrose (0.1 ml, 300 mM). Following a preincubation period (5 min at 35 °C), the assay was started by the addition of the enzyme solution (0.1 ml, 1 mg ml^{-1}) and incubation was continued for 5 min. In order to terminate the enzymatic reaction, the reaction medium was then placed in a boiling water bath for 5 min.

The same assay medium was used to determine the activity of the immobilized enzyme. The enzymatic reaction was started by the introduction of 10 membrane disks into the assay medium (10 ml) and was carried out at 35 °C with shaking in a water bath. After 15 min, the reaction was terminated by removal of the membrane disks from the reaction mixture.

Sucrose hydrolyses by the free and immobilized preparations were determined by measuring the glucose content of the medium according to a method described previously (Sigma Technical Bulletin No. 510, 1983). 2.5 ml of assay mixture (containing GOD 25 mg, POD 6.0 mg, and *o*-dianisidine, 13.2 mg in phosphate buffer 100

ml, 0.1 M, pH 7.0), and 0.1 ml of enzymatically hydrolyzed sample were mixed and then incubated in a water bath at 35 °C for 30 min. After addition of sulphuric acid (1.5 ml, 30%), absorbance was measured in a UV–visible spectrophotometer (Shimadzu, model 1601, Tokyo, Japan), at 525 nm.

The activities of the free and immobilized invertase were expressed in μmol sucrose min^{-1} mg^{-1} of enzyme and μmol sucrose min/cm^{-2} of enzyme membrane. These activity assays were carried out over the pH range 4.0–8.0 and temperature range 20–60 °C to determine the pH and temperature profiles of the free and the immobilized enzyme. The results of dependence of pH, temperature, storage stability, and repeated runs are presented in a normalized form with the highest value of each set being assigned the value of 100% activity.

2.6. Kinetic parameters K_m and V_{max}

K_m and V_{max} values of the free enzyme were determined by measuring initial rates of the reaction with sucrose (0–300 mM) in acetate buffer (50 mM, pH 5.0) at 35 °C. The kinetic parameters of immobilized invertase were determined in a batch system by varying the concentrations of sucrose (0–300 mM) in acetate buffer (50 mM, pH 5.0). The reaction temperature was 35 °C. K_m and V_{max} values were calculated from the data obtained after 15 min.

2.7. Storage stability

The activities of the free and the immobilized invertase after storage in acetate buffer (50 mM, pH 5.0) at 4 °C was measured in a batch operation mode.

2.8. Thermal stability

The thermal stabilities of the free and the immobilized invertase were carried out by measuring the residual activity of the enzyme exposed to three different temperatures (50–70 °C) in acetate buffer (50 mM, pH 5.0) for 2 h. After every 15 min time interval, a sample was removed and assayed for enzymatic activity.

2.9. Water content of pHEMA–GMA membranes

The water content of membranes were determined in distilled water. Dry membrane pieces were placed in distilled water at 25 °C until they reached a constant weight for about 4 h. Swollen membrane was weighed by an electronic balance (Shimadzu, Japan, EB. 280).

$$\text{Water content } \% = [(Ws - Wo/Wo)].100$$

The water contents of pHEMA–GMA membranes were calculated using the above expression, where Ws and Wo are weights of swollen and dry membrane, respectively.

3. Result and discussion

3.1. pHEMA–GMA membranes

The water contents of pHEMA–GMA membranes were found about 49.3%. The surface structure of a membrane has a large external surface area and this is important for the immobilization of the proteins. Schematic representation for the formation of pHEMA–GMA membranes and invertase immobilization onto membranes is shown in Fig. 1.

3.2. Immobilization of invertase onto PHEMA–GMA membrane

Invertase was immobilized by the condensation reaction of the epoxy groups of glycidyl methacrylate into the membrane structure with amino groups of the enzyme (Fig. 1). During the condensation reaction, amide bonds are formed between epoxy groups of the support and amino groups of the enzyme. The enzyme loading and the retained enzyme activity, after immobilization on the pHEMA–GMA membrane, were 102 μg cm^{-2} and 40%, respectively.

3.3. K_m and V_{max}

K_m and V_{max} values were significantly affected after covalent immobilization of invertase onto the pHEMA–GMA membranes. (Table 1). The K_m values for free and immobilized enzyme were estimated from Lineweaver–Burk Plots. The value of K_m for covalently bound invertase was approximately 2.6-fold higher than that of free enzyme. In a similar study, Prodanovic, Javonovic and Vujcic (2001) reported that the K_m values of covalently bound invertase via its carbohydrate moiety onto glycidyl methacrylate membranes were 1.5 times higher than that of free invertase. These changes in the affinities are probably caused by the lower accessibility of the substrate to the active site of the immobilized enzyme.

Table 1
Kinetic parameters of the free and immobilized invertase

Types of enzyme	K_m (μM) ×1000	V_{max} (μmol/min)	Recovered activity (%)
Immobilized invertase	58	26.8	60
Free invertase	22	44.8	100

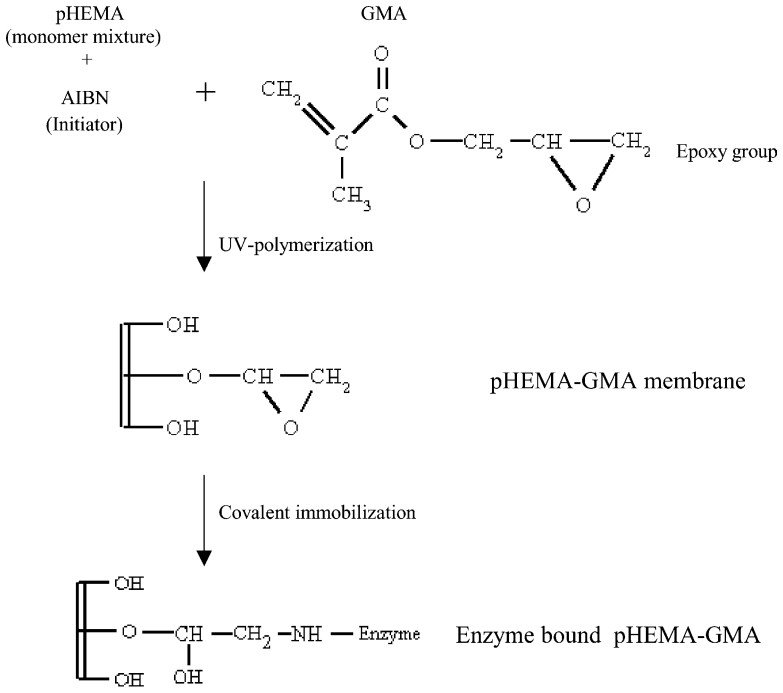

Fig. 1. Schematic representation for the formation of pHEMA–GMA membranes and invertase immobilization onto membranes.

The V_{max} value decreased by approximately 40% with respect to free enzyme after immobilization. The V_{max} of invertase immobilized on the porous pHEMA–GMA membrane can be affected by external or internal diffusional resistances. This resistances can include external and internal transport of the substrate and products to the surface of the membrane.

3.4. Effect of temperature on the activity optimum

For the determination of the catalytic activities of the free and the immobilized invertase, studied in 50 mM, pH 5.0 acetate buffer in the temperature range 25–70 °C (Fig. 2). The optimum temperature for the immobilized and free invertase were 55 °C and 45 °C, respectively. Increase in optimum temperature was caused by changing physical and chemical properties of the enzyme. The covalent bond formation, via epoxy groups of the immobilized invertase, reduces the conformational flexibility, resulting in higher activation energy for the molecule to reorganize to a proper conformation for substrate binding (Desmukh, Shoudhury, & Shankar, 1993; Gupta, 1991).

3.5. Effect of pH on the activity

Studied pH values for the free and immobilized invertase activities were in the pH range 3.0–8.0 (Fig. 3). Reactions were carried out in acetate and phosphate buffers. The optimum pH value for the free invertase was obtained at pH 5.0. The optimum pH of the

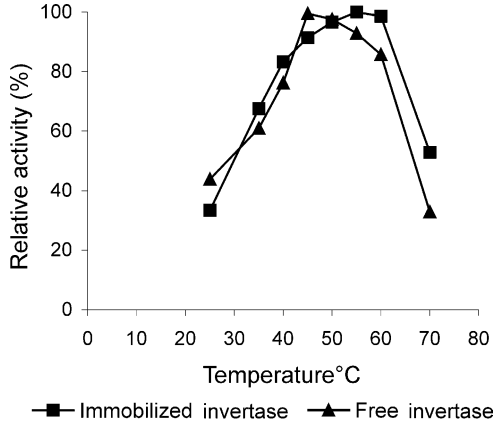

Fig. 2. Temperature profiles of the free and the immobilized invertase.

immobilized enzyme was shifted 0.5 pH unit to the alkaline region. This shift is possibly due to the secondary interactions between the enzyme and the polymeric matrix. (Arıca, Denizli, Baran, & Hasırcı, 1998). Other researchers (Erginer, Toppare, Alkan & Bakır, 2000; Mansfeld et al., 1991; Tien & Chiang, 1999) have obtained similar results upon immobilization of invertase and other enzymes.

3.6. Storage stability

The free and immobilized invertase preparations were stored in pH 5.0 acetate buffer at 4 °C. Then activities were measured over a period of 35 days (Fig. 4). The free enzyme lost all its activity within 32 days. Immobi-

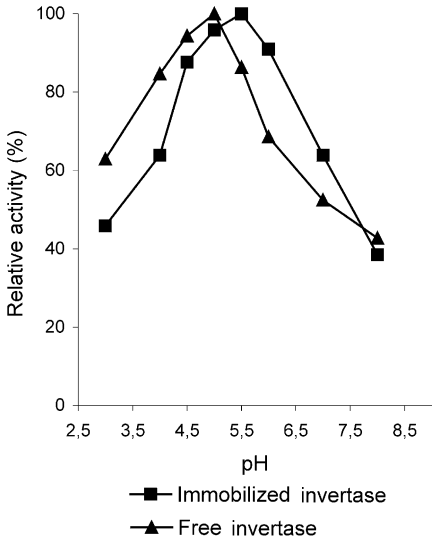

Fig. 3. pH profiles of the free and the immobilized invertase.

lized enzyme lost about 22% of its activity during the storage period.

3.7. Thermal stability

Thermal stability studies of free and immobilized invertase were carried out at various temperatures (Fig. 5). While immobilized invertase was stable at 50 °C, the free form retained about 90% of its initial activity during a 90-min incubation period. At 60 °C the free and immobilized enzymes retained about 56 and 19%, respectively. According to these results, the immobilized form was inactivated at a slower rate than the free form. Half-lives of free and immobilized invertase were 11 and 38 min at 70 °C, respectively. Similar observations have been previously reported for various immobilized systems (Germain & Crichton, 1986; Ulbrich, Shellenberger & Damerau, 1986;).

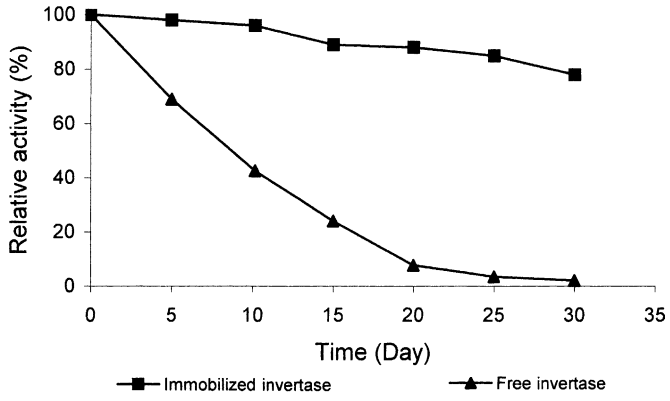

Fig. 4. Storage stabilities of the free and the immobilized invertase.

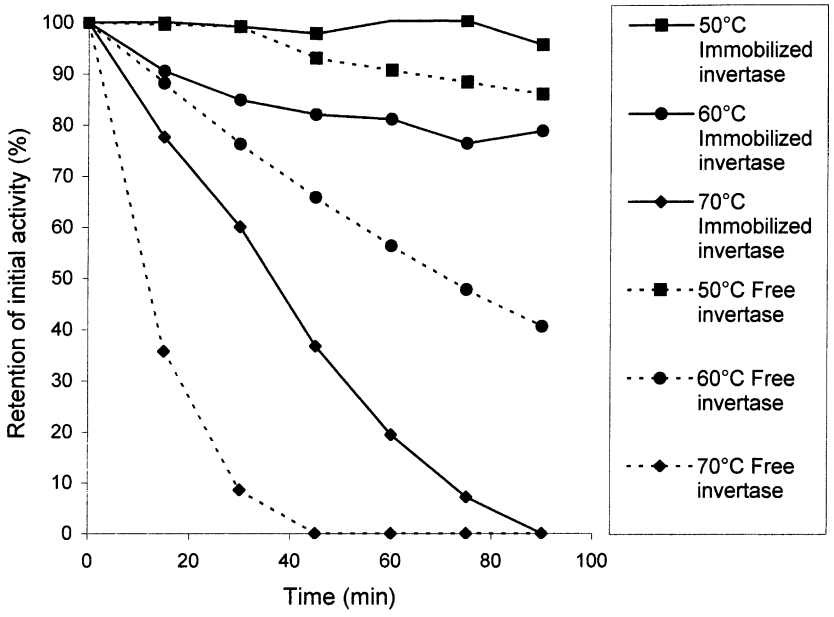

Fig. 5. Thermal stability of the free and the immobilized invertase.

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