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The effect of gel composition on the adsorption of invertase on poly(acrylamide/maleic acid) hydrogels

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Abstract

The effects of external stimuli such as pH of solution, temperature, substrate concentration of solution and storage stability on the invertase adsorption capacity of poly(acrylamide/maleic acid) [P(AAm/MA)] hydrogels, synthesized by gamma irradiation of ternary mixtures of AAm/MA/Water, were investigated. The adsorption capacities of the hydrogels were found to increase from 4.0 to 13.3 mg invertase/g dry gel with increasing amount of MA in the gel system, while P(AAm) gel adsorbed only 3.1 mg invertase/g dry gel. Kinetic parameters were calculated as 20.6 mM for K_m and 6.44×10^{-5} mol/dm^3 min for V_{max} for free enzyme and in the range of 23.6–57.7 mM for K_m and 8.62×10^{-5}–1.05×10^{-4} mol/dm^3 min for V_{max}, depending on the amount of MA in the hydrogel. Enzyme activities were found to increase from 50.0 to 74.0% with increasing amount of MA in the gel system and retained their activities for one month storage. The enzyme activities, after storage at 4°C for one month, were found to be 21.0 and 50.0–74.0% of the initial activity values for free and adsorbed enzyme, respectively. The optimal pH values for free and adsorbed enzymes were determined as 4.56 and 4.56–5.00. The optimum temperature for free and adsorbed enzymes was 55°C. Adsorption studies show that, not only gel composition but also the stimuli, temperature and pH of the solution, play important roles on the invertase adsorption capacity of poly(AAm/MA) hydrogels. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Invertase; Immobilization; Adsorption; Poly(acrylamide/maleic acid) hydrogel

1. Introduction

Hydrogels are water-swollen, crosslinked polymeric structures produced by the simple reaction of one or more monomers, which find an extremely wide range of applications in the fields of medicine, pharmacy, biotechnology, agriculture and controlled release of drugs. In recent years hydrogels have been used for the immobilization of enzymes, proteins, antibodies and antigens, due to their versatile applications in biomedicine and biotechnology (Mathias & Alexandra, 1997; Monsan & Combes, 1984; Saraydın, Karadağ, Öztop & Güven, 1994; Peppas, 1986). The possibilities and advantages of using hydrogels for immobilization of enzymes have been widely described in the literature. General operational advantages of immobilized enzymes are reusability, possibility of batch or continuous operational modes, rapid termination of reactions, controlled product formation and easy separation of the product, a great variety of engineering design for continuous processes and possible greater efficiency in consecutive multistep reactions.

There are many reactions of interest for industry where immobilized enzymes are used as catalysts. Most biochemical catalysts are used for conversion of biological materials, but there is growing awareness of their potential applications in chemical conversions. The choice between use of soluble free and immobilized enzymes depends on the cost the enzyme, the nature of the conversion process, and the relative operational stabilities of the two forms. By their nature, some food processes such as meat-tenderization and baking, involve the addition of the enzymes at the final processing stage, making reuse impossible. Sometimes the ability to remove the immobilized enzyme from the product stream, ensuring minimal contamination by protein, may influence the choice but the main factor is the operational stability of the enzyme. As long as the

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enzyme can be stabilized by modification or immobilization, reuse of the enzyme may be worthwhile (Kennedy & Cabral, 1987).

Sucrose, commonly known as table sugar, is a disaccharide composed of an alpha-D-glucose molecule and a beta-D-fructose molecule linked by an alpha-1,2-glycosidic bond. When this bond is cleaved in a hydrolysis reaction, an equimolar mixture of glucose and fructose is generated.

The invertase (EC 3.2.1.26) enzyme which catalyzes the hydrolysis of sucrose to glucose and fructose is largely used in the food industry to prevent the crystallization of sucrose in sugar mixtures. For example, honey contains a significant percentage of invert sugar, namely D-glucose and D-fructose, resulting from the hydrolysis of sucrose using the enzyme invertase. A number of studies have been reported in the literature on the immobilization and adsorption of invertase on various polymeric agents and gels. Invertase has been immobilized on diazotized 4-aminobenzoylcellulose. The optimum coupling conditions namely enzyme concentrations, time and pH have been determined (Şen, Pekel & Güven, 1998; Simionescu, Popa & Dumitru, 1987).

The copolymer used for the preparation of the hydrogel in this study is P(AAm/MA), which is one of the most hydrophilic and highly biocompatible copolymers. In our previous studies, we had reported on P(AAm/MA) copolymeric hydrogels synthesized by a radiation-induced polymerization technique (Tümtürk, Çaykara, Şen & Güven, 1999). In this study, we report the enzymatic performance of these hydrogels at various temperatures and pH values and compare them with free enzyme behaviour.

2. Materials and methods

2.1. Chemicals

Acrylamide (AAm) and maleic acid (MA) were obtained from Fluka and Merck. The invertase (fructofuranosidase, E C 3.2.1.26) used for the adsorption studies was obtained by Fluka. Sucrose was a product of Sigma and used as the substrate. Chemicals used in the preparation of buffers were supplied by Merck and used without further purification. Glucose of biochemica-grade was provided by Fluka.

2.2. Preparation of hydrogels

Aqueous solutions of monomers of 1 g AAm and 20, 40, 60 and 90 mg MA were prepared in 1 ml of pure water in different compositions (AAm/MA mole ratios, 100:0.0, 98.8:1.2, 97.6:2.4, 96.5:3.5, 94.8:5.2). Monomer solutions, thus prepared, were placed in the PVC straws of 4 mm diameter and irradiated to 25 kGy in air at ambient temperature in gamma cell 220 type γ irradiator at a fixed dose rate of 0.17 kGy/h. Hydrogels obtained in long cylindrical shapes were cut into pieces of 3–4 mm long. The amount of MA in the monomer, polymer and/or copolymer form was determined by titration of extract against NaOH (0.05 M) to phenolphthalein endpoint.

2.3. Adsorption

For the adsorption of invertase in P(AAm/MA) hydrogels, 0.1 g of the dry gel was placed in 20 ml solution of 40 mg dl^{-1} invertase and the adsorption was carried out at 30°C in a shaking waterbath for 8 h. Hydrogels were separated and the unadsorbed enzyme was removed by washing with phosphate buffer (15 ml). The adsorbed enzymes were used freshly or stored at 4°C.

2.4. Activity of invertase adsorbed in hydrogels

The enzymatic activity of the adsorbed invertase was determined by an enzyme reaction using sucrose as substrate. The adsorbed and free invertase activities were determined by Folin-Wu Method assay solution containing free or adsorbed enzyme in phosphate buffer (0.2 mg enzyme or 0.1 g copolymer in 1.0 ml phosphate buffer) placed in a test tube. Substrate solution (1% sucrose) was added into the tubes and incubation continued for exactly 15 min. At the end of 15 min the tubes were removed from the water bath (30°C). One millilitre of alkaline copper sulphate solution was added to the tube to terminate the reaction. Then the tube was placed in the boiling water bath until the observed brown colour developed and then cooled in water at room temperature. Then 1 ml of phosphomolybdic acid reagent was added into the tube and mixed thoroughly by vortex. Finally, 10.0 cm^3 of phosphate buffer were added. The absorbance was read photocolorimetrically at 640 nm versus a blank solution, which was prepared in the same manner, but lacking the enzyme. The amount of glucose was obtained from the calibration curve and used in the calculation of enzyme activity. The activity of invertase as mol of (glucose + fructose) formed/mg protein/min was calculated (1U of enzyme activity is defined as that amount of enzyme which hydrolyses 1 mol sucrose/min under the present assay conditions).

2.5. Enzyme adsorbed efficiency

After the enzyme adsorption process and separation of poly(AAm/MA) hydrogels, the supernatant and the wash solutions were collected and UV absorbance of solutions was measured at 280 nm by using a Jenway 5105 UV-vis spectrophotometer. The amount of unadsorbed enzyme was calculated from the enzyme calibration curve which was obtained at the same wavelength.

3. Results and discussion

3.1. Preparation of hydrogels

In this study, ionizing radiation was used for the preparation of AAm/MA hydrogels. When an aqueous solution of the monomer, AAm and MA, is irradiated with ionizing radiation, such as gamma rays, free radicals are generated not only from the monomer, but also even more efficiently in the solvent water. The reaction of these radicals with the monomers leads to the formation of random copolymers of AAm/MA. When the radiation dose has been increased beyond a certain value, the polymer chains crosslink and gel is then obtained. The total dose required for 100% gelation of AAm/MA hydrogels has been found to be 7 kGy when MA used in the range of 0.0–0.35 mol% in the initial mixture. In this study, for the preparation of mechanically-stable hydrogels, the ternary mixtures of AAm/MA/H_2O were irradiated to 25 kGy with gamma rays. Mol percentages of MA in the p(AAm/MA)-1, p(AAm/MA)-2 p(AAm/MA)-3 p(AAm/MA)-4 hydrogels are 1.1, 1.2, 3.1 and 4.5, respectively. Swelling behaviour, diffusional properties, and network properties of these hydrogels were investigated in our previous study.

3.2. Enzyme loading

The amount of enzyme which was loaded with the hydrogels was 4.0–13.3 mg invertase/g dry gel while P(AAm) adsorbed 3.1 mg invertase/g dry gel. The total amount of adsorbed invertase depends on the mol percentage of MA in the gel system and is given in Fig. 1. As shown, an increasing the amount of MA from 1.1 to 4.5 mol% causes a three-fold increase in the adsorbed invertase content. The reason for this increase can be thought of as an increase in specific interaction between positively charged enzyme molecules and ionized MA in the hydrogel as well as an increase of free volume of gel available for diffusion. For invertase-adsorbed systems, various adsorbed capacities are reported as 2.8 and 3.5 mg/g on poly(ethylene–vinyl alcohol) membrane supports (Imai, Shiami, Uchida & Miya, 1986). The adsorbed capacity values found in this study are higher than the values in the literature.

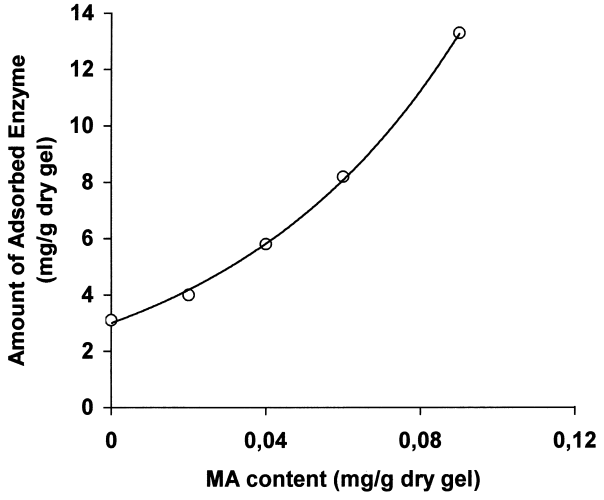

Fig. 1. The variation of adsorbed invertase with MA content in the gel system.

3.3. Effect of pH and temperature on enzyme activity

The enzymatic activities were estimated, for both free and adsorbed invertase, by measuring the absorbance at 640 nm when reactions were carried out at various pH values, temperatures, substrate concentrations and storage times. The influence of pH on the enzyme activity was studied in the pH range 3.0–8.0 at 30°C. The pH activity profiles of invertase adsorbed on the hydrogels were similar in both situations (Fig. 2). The pH for maximum substrate conversion was determined as 4.56 for free invertase and 4.56–5.00 for adsorbed invertase, irrespective of the MA content in the hydrogel. The optimum pH values of free immobilized invertase are reported in the literature to be between 4.0 and 5.4 (Paul, Li & George, 1977). The shift in the optimum pH toward a lower value upon adsorption could be due to the difference in the hydronium ion concentrations between the gels and the bulk solution. Poly-ionic matrices causing partitioning of protons between the bulk phase and enzyme microenvironment are well known (Arıca, Hasırcı & Alaeddinoğlu, 1995).

The effects of temperature on the activity of free and adsorbed invertases are given in Fig. 3. As can be seen, the adsorbed invertase showed the same activity dependence on the temperature as the free enzyme. Besides, the

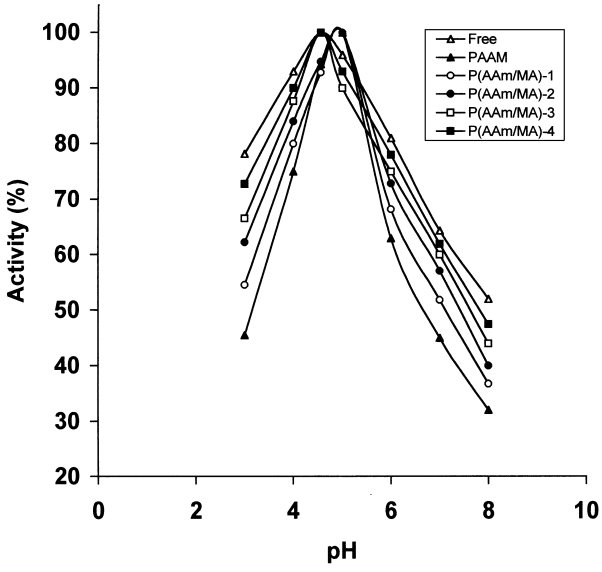

Fig. 2. Effect of pH on free and adsorbed invertase activity.

optimum temperatures of the adsorbed systems were found to change very much with increasing amount of MA in the gel system. This behaviour was attributed to the creation of conformational limitations on the enzyme movement as a result of lower interaction between the enzyme and the matrix. For free and immobilized invertase systems the same temperatures have been reported (Farahdiba & Saleemuddin, 1997; Simionescu et al., 1987).

3.4. Kinetic parameters

The effect of substrate concentration on the hydrolysis of sucrose by free and hydrogel-adsorbed invertase was investigated. The results of these experiments, presented in Fig. 3 by the method of Lineweaver-Burk show that the hydrogel-supported invertase preparations obey Michaelis-Menten kinetics. The Michaelis-Menten

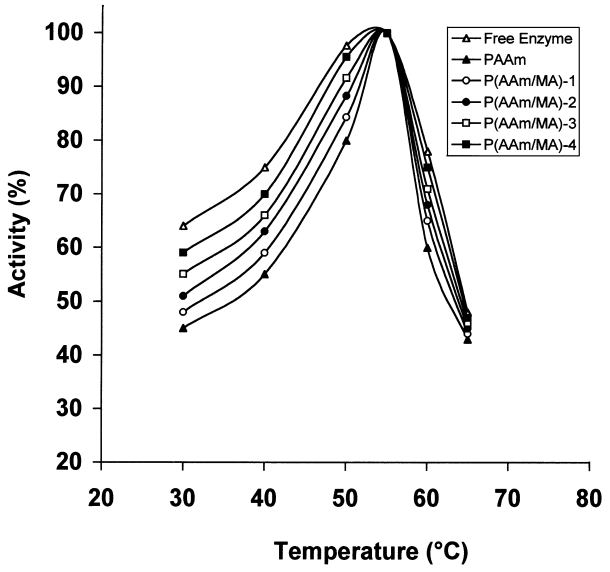

Fig. 3. Effect of temperature on free and adsorbed invertase activity.

Table 1
Values of K_m and V_{max} for the hydrolysis of sucrose by invertase in free solution and adsorbed on P(AAm/MA) hydrogels

Gel name	K_m (mM)	V_{max} (mol dm^{-3} min)$\times 10^5$
Free invertase	20.6	6.44
PAAm	57.7	13.50
P(AAm/MA)-1	42.0	10.50
P(AAm/MA)-2	32.0	8.62
P(AAm/MA)-3	27.9	7.81
P(AAm/MA)-4	23.6	7.02

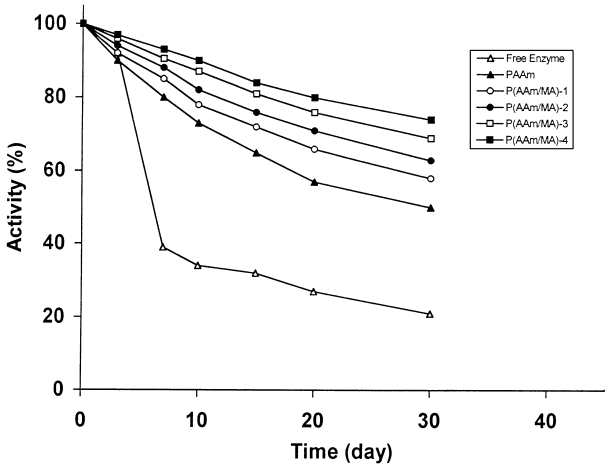

Fig. 5. Storage stability of free and adsorbed invertase at 4°C.

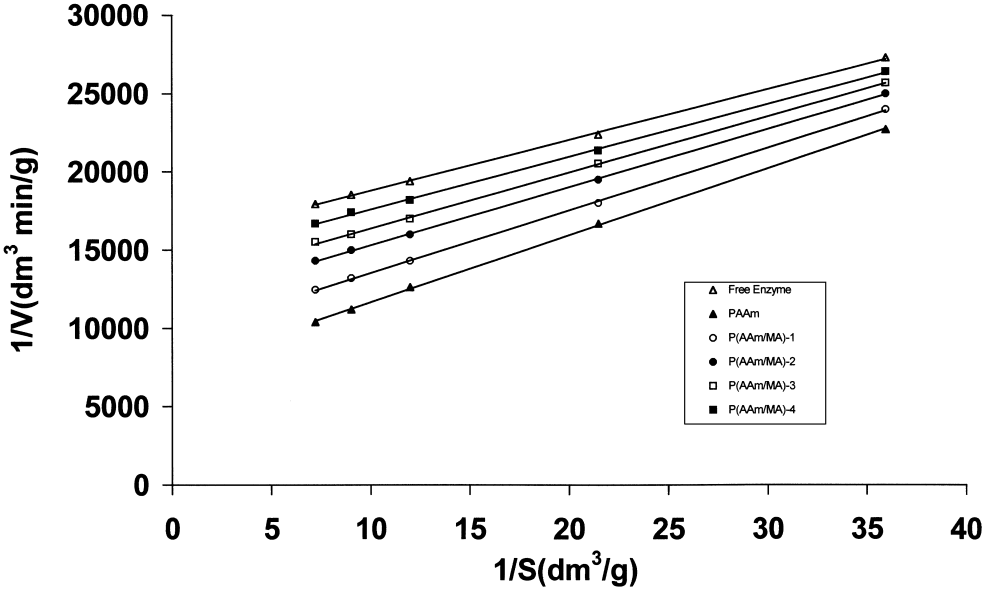

Fig. 4. Lineweaver-Burk plots for free and adsorbed invertase.

Table 2
Activity values of invertase studied in storage stability[a]

Time (day)	Free invertase	P AAm	P (AAm/MA)-1	P (AAm/MA)-2	P (AAm/MA)-3	P (AAm/MA)-4
0	3.66×10^{-5}	1.14×10^{-4}	1.46×10^{-4}	2.12×10^{-4}	3.00×10^{-4}	4.87×10^{-4}
3	3.37×10^{-5}	1.02×10^{-4}	1.35×10^{-4}	2.00×10^{-4}	2.88×10^{-4}	4.72×10^{-4}
7	1.43×10^{-5}	0.91×10^{-4}	1.24×10^{-4}	1.87×10^{-4}	2.72×10^{-4}	4.53×10^{-4}
10	1.24×10^{-5}	0.83×10^{-4}	1.14×10^{-4}	1.74×10^{-4}	2.61×10^{-4}	4.38×10^{-4}
15	1.17×10^{-5}	0.74×10^{-4}	1.05×10^{-4}	1.61×10^{-4}	2.43×10^{-4}	4.09×10^{-4}
20	0.99×10^{-5}	0.65×10^{-4}	0.97×10^{-4}	1.51×10^{-4}	2.28×10^{-4}	3.89×10^{-4}
30	0.77×10^{-5}	0.57×10^{-4}	0.85×10^{-4}	1.34×10^{-4}	2.07×10^{-4}	3.60×10^{-4}

[a] Unit of activities: mol dm^{-3} min^{-1}.

Table 3
The amounts of invertase absorbed on polymeric hydrogels[a]

Time (day)	P AAm	P (AAm/MA)-1	P (AAm/MA)-2	P (AAm/MA)-3	P (AAm/MA)-4
0	3.10	4.00	5.80	8.20	13.3
3	2.79	3.68	5.45	7.87	12.9
7	2.48	3.40	5.10	7.42	12.4
10	2.26	3.12	4.76	7.13	12.0
15	2.02	2.88	4.41	6.64	11.2
20	1.77	2.64	4.12	6.23	10.6
30	1.55	2.32	3.65	5.66	9.84

[a] mg Invertase/0.1 g dry gel.

constant, K_m, with the small substrate, maltose, were 3.5 $mmol^{-1}$ l for free and 3.2 $mmol^{-1}$ l for covalently bound AG whereas V_{max} values with maltose and starch dropped to 32 and 22%, respectively, for the immobilized versus free enzyme (Mathias & Alexander, 1997). In this study, the values for the Michaelis-Menten parameters of free and adsorbed invertase, as calculated from the results shown in Fig. 4, are shown in Table 1. These results show that the K_m with respect to sucrose is increased over two-fold depending on the amount of MA in the hydrogel. However, they were calculated as 20.6 mM for K_m and 6.44×10^{-5} mol/dm^3 min for V_{max} for free invertase (Table 1). This indicates that formation of the enzyme–substrate complex is more difficult with the adsorbed invertase (Simionescu et al., 1987). However, this increase may be a consequence of either structural changes in the enzyme, introduced by the applied adsorption procedure, or lower accessibility of the substrate to the active site of the adsorbed enzyme.

3.5. Storage stability

Enzymes are not stable in solutions and during storage their activities decrease with time. When invertase solution is stored at 4°C activities, after storage for one month, were found to be 21% and 50–74% of the initial activity values for free and adsorbed, depending on the amount of MA content in the hydrogel, respectively (Fig. 5). Stability of adsorbed invertase with increasing MA content in the hydrogel showed significant increase, especially at longer storage periods. This can be attributed to the increase of specific interactions between positively charged enzyme molecules and ionized hydrogel. For the storage stability studies of immobilized and free invertase, the activity values observed after every usage are given in Table 2. The amounts of invertase adsorbed onto the polymeric gel composition, in the same studies, are given in Table 3.

4. Conclusion

In this study, we have tried to see the effect of gel composition on the invertase adsorption capacity of P(AAm/MA) hydrogels. Based on an experimental programme, the following optimum values of the reaction parameters for storage stability, pH and temperature, respectively were found; 50–74% of the initial activity, 4.56–5.00 and 55°C, depending on the amount of MA content in the hydrogels. Free and adsorbed invertase has the same optimum reaction pH and temperature. The reactions catalyzed by the adsorbed invertase have a larger apparent Michaelis constant MA content in the hydrogel system, as is clearly shown in this work, in the invertase solutions plays an important role on the adsorption behavior of P(AAm/MA) hydrogels; these hydrogels are potential sorbents for the enzyme invertase.

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