A Hydrogel Capable of Facilitating Polymer Diffusion through the Gel Porosity and Its Application in Enzyme Immobilization

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ABSTRACT: A hydrogel was designed and prepared which is capable of promoting the diffusion of soluble starches through its pores. The preparation was carried out via the iterative freezing–thawing of an aqueous poly(vinyl alcohol) solution containing concanavalin A, Con A, which had a binding affinity toward the starch to be diffused through the gel porosity. Gel permeation chromatographic analysis of the starch remaining in an aqueous solution after shaking with the Con A-loaded gel showed that the starch concentration of the gel phase was effectively enhanced. This strongly suggests that an attractive force (biochemical affinity) between the Con A within and the starch outside of the gel facilitated the diffusion of the starch through the gel porosity. The present Con A/gel system was applied in the immobilization of glucoamylase which was performed through the physical gel entrapment of both the enzyme and Con A. The gel with the entrapped enzyme hydrolyzed starch (as a polymeric substrate) into glucose in an aqueous system. Increased enzyme activity was observed due to the facilitated diffusion of the substrate induced by the Con A.

Introduction

Since the original use of a gel, cross-linked poly(acrylamide), for the immobilization of enzymes through physical entrapment by Bernfeld and Wan,[1] hydrogels have been frequently employed to immobilize enzymes or other biopolymers. We have recently performed the gel entrapment of an enzyme[2] or a lectin[3] to construct a novel immobilized system in which the gel (as the supporting matrix) itself swells and shrinks in response to biochemical changes induced by the protein molecules within the gel phase. Using the gel-entrapment method, we may take advantage of the great resistance to polymer diffusion due to gel porosity. However, this limited diffusion within the gel phase also causes a reduced mass transfer rate for low molecular-weight compounds, which diminishes the chances for collision and reaction between the molecules lying within the gel and coming from the external bulk phase. For example, in immobilized enzyme reactions, the mass transfer resistances for substrate and product within the pores of the gel are generally responsible for a loss in activity upon immobilization.[4]

Two approaches have been reported to facilitate the pore diffusion of solutes (i.e., the diffusion of solutes through the gel porosity) in hydrogel systems.[5,6] The first involves the cyclic pumping of the surrounding medium by the gel during the physically controlled collapse and expansion of its polymer chain network. Hoffman et al. have reported the preparation of a functional hydrogel, consisting of a lightly cross-linked copolymer of *N*-isopropylacrylamide and acrylamide,[5] which shrinks when warmed above a critical temperature but reswells when cooled below that temperature. This shrinking and swelling process can be repeated reversibly by means of the thermal cycling operation to increase the mass transfer rates of solutes from the outside to the inside of the gel, and vice versa. This cyclic operation thus enhances the overall activity of the gel-entrapped enzymes when the system is applied in immobilized enzyme reactions.

The second approach is based on the gel entrapment of a polymeric carrier which has a high binding affinity toward the solute to be diffused through the pores.[6] Such an affinity may result in the development of an attractive force between the carrier inside and the solute outside the gel and thereby promote pore diffusion. We have reported that the physical gel entrapment of a sugar-binding protein, caster bean lectin (CBL), promotes the uptake of *O*-nitrophenyl β-D-galactopyranoside (ONPG) by the gel via pore diffusion. In addition, this lectin-facilitated pore diffusion makes an important contribution to the activation of the enzymatic hydrolysis of ONPG in a hydrogel into which β-D-galactosidase is coimmobilized with CBL. However, both of these previous gel systems involved low molecular-weight solutes. Attempts to promote the pore diffusion of polymeric solutes have not yet succeeded, especially for immobilized enzyme reactions.

The present paper reports that our approach[6] is effective not only for the promotion of the diffusion of a polymer solute (soluble starch, SS) through the gel porosity but also for the activation of glucoamylase immobilized within a hydrogel. Concanavalin A (Con A), a lectin derived from jackbeans, was selected as the carrier in view of its strong binding affinity toward sugar molecules or sugar residues in polysaccharides.[7] A hydrogel prepared from poly(vinyl alcohol) (PVA) by the iterative freezing–thawing method was used in the immobilization of Con A and the coimmobilization of Con A with the enzyme.

Experimental Section

Materials. PVA (degree of polymerization = 2000) and SS were of commercial origin (Wako Pure Chemical Industries, Ltd.). All proteins were the same samples used previously:[3,8,9] human serum albumin (HSA), $M_w = 6.6 \times 10^4$;[8] Con A, $M_w = 5.2 \times 10^4$;[3,9] and glucoamylase (or *exo*-1,4-α-D-glucosidase, EC.3.2.1.3), $M_w = 1.5 \times 10^5$.[9]

Gelation and Protein Immobilization. A citrate buffer (50 mM, pH 5.0) was used as the solvent. PVA powder (1.8 g) was suspended in about 10 g of the solvent and completely dissolved by heating. The polymer concentration was then adjusted to 18% (w/w) with a slight amount of distilled water. The viscous polymer solution obtained was mixed well with 2 g of the solvent or solutions containing the proteins (132 μg for the enzyme and 7.2 mg for the other proteins) and then transferred into the cylindrical holes (3 mm diameter and 2 mm depth) of a plastic container. Gelation was carried out by the iterative freezing–thawing method; the polymer solution was frozen by soaking the container in *n*-hexane precooled at −20 °C for 12 h, followed by thawing at room temperature for 3 h. Such freezing and thawing procedures were further repeated six times at suitable time intervals (ca. 3 h each). The gelled polymer was finally removed from the container and thoroughly washed with the solvent used in the preparation. The protein content of each gel preparation was calculated from the amounts of the proteins released from

Table I
Contents of Protein in PVA Gel-Immobilized Preparations[a]

immobilized preparation	abbrevn	protein content (μg/g of gel)[b] total	protein content (μg/g of gel)[b] enzyme
gel with:			
immobilized concanavalin A	IC	498	0
immobilized human serum albumin	IH	503	0
immobilized glucoamylase	IG	9.1	9.1
coimmobilized glucoamylase with concanavalin A[c]	CIGC	507, 501[d]	9.0
coimmobilized glucoamylase with human serum albumin	CIGH	511	9.3

[a] The immobilization was repeated several times under the same conditions, because the amounts of the proteins released from the same kind of preparation differed somewhat from one another; the sample for which the contents of the protein and/or the enzyme were very close to those of the other kinds when then chosen and used for the measurements. [b] All data are expressed on the basis of the weight for the initially prepared wet gel. [c] The immobilization was carried out using an aqueous polymer solution (12 g) containing 18% (w/w) PVA, 108 μg of glucoamylase, and 6 mg of Con A, because there was no protein released from the gel preparation during the washing process (see ref 10). [d] This sample was used to confirm that the activity of CIGC is not due to the leakage of enzyme from the gel.

the gels during the washing process.[10] The protein concentrations of the washing extracts were determined by a combination of the following two analytical methods, as carried out in a previous study:[9] dye-binding assay with Coomassie Brilliant Blue G-250 for the total protein concentration;[11] and assay of glucoamylase activity for the enzyme concentration (see below). The protein contents of the gel samples are summarized in Table I, together with the abbreviations used in this paper.

Gel Permeation Chromatography (GPC). The GPC curves were measured to study differences in the uptake of SS by the gels with and without Con A. The gel (1 g) was soaked and stirred in 2 mL of an aqueous 3% (w/v) SS solution at 25 °C for 12 h. SS remaining in the supernatant solution was then analyzed using a Hitachi 635 A liquid chromatograph equipped with both a Shodex RI/SE-11 detector and a YMC Diol-300 column. Well-degassed distilled water was used as the eluent.

Enzyme Reaction and Assay of Enzyme Activity. The gels (2 g) with the immobilized enzyme were gently stirred with a substrate solution (2 mL) containing 0.125 to 1% (w/v) of SS and thermostating at 37 °C. Glucose produced enzymatically from SS was analyzed by a mutarotase–glucose assay method.[12] Two buffer solutions were used for maintaining pH during the reaction: a 50 mM citrate buffer (pH 3–6) and a 50 mM phosphate buffer (pH 7–8).

Results and Discussion

We first examine the effect of the entrapment of Con A on the uptake of SS by the PVA gel. Next, we investigate the hydrolysis of SS by glucoamylase which had been immobilized or coimmobilized with Con A within the gel. Finally, we discuss the role of Con A-induced facilitated pore diffusion of SS in the immobilized enzyme reaction by examining the kinetic parameters of the native and gel-entrapped enzymes.

Effect of Con A on Starch Uptake by Gel. By measuring the equilibrium distribution between the gel and bulk phases of a solute, the characteristics of its diffusion through the gel porosity can be studied. In the present system, however, such a tool was not available because the diffusion of polymers through the gel pores was subject to different steric hindrance effects depending upon their molecular sizes. Thus, we employed the GPC method for studying the diffusibility of SS molecules through the pores of the PVA gel.

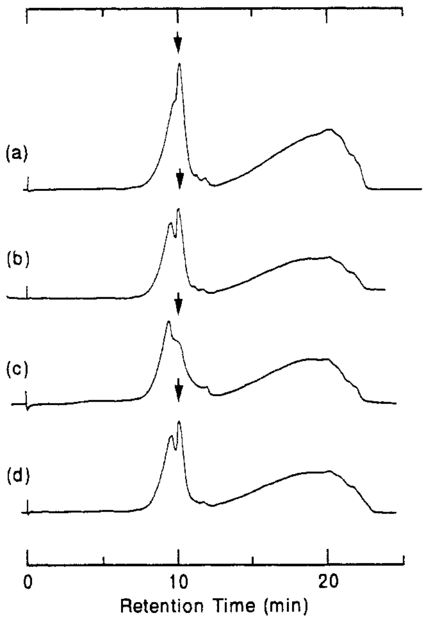

Figure 1. GPC curves of the original SS (a) and of the remaining SS after its diffusion into different PVA gels: (b) PVA gel without the proteins; (c) IC; and (d) IH. The vertical axis denotes the intensity of an RI detector which is directly proportional to SS concentration.

As can be seen from the GPC curves in Figure 1, the original SS had two main peaks: a sharp peak ($P_{t=10}$) with a shoulder at a retention time of 10 min and a broad peak ($P_{t=20}$) at a retention time of 20 min. SS is known to be a branched polymer consisting of two carbohydrates with different molecular weights:[13] amylose ($M_W = 3 \times 10^4$–1.7×10^5) in which D-glucose units are linked in a linear fraction by α-1,4 linkages and amylopectin ($M_W = 5 \times 10^4$–5×10^8) as a branched polysaccharide in which shorter chains of glucose units linked α-1,4 are also joined to each other by α-1,6 linkages. It is extremely difficult to determine the molecular weight for such a natural polymer from the GPC data, because we cannot obtain a standard sample for making a calibration curve. However, we believe it to be a general rule in CPC that a high molecular-weight polymer is eluted more rapidly from a column than a low molecular-weight one. Therefore, we were able to assign $P_{t=10}$ to a high molecular-weight fraction and $P_{t=20}$ to a low molecular-weight fraction.

It can be observed from Figure 1 that $P_{t=10}$ splits into two spikes when the SS remaining in an aqueous solution after shaking with the original PVA gel (not containing Con A) was subjected to GPC analysis. This phenomenon is related to the well-known molecular sieve effect of polymer gels. When the gel (IC) with immobilized Con A was used instead of the original gel, the molecular sieve effect was further strengthened, and one of the two split peaks became a shoulder (see arrow in Figure 1). Therefore, it is apparent that the immobilized Con A contributes to the enhancement of the uptake of SS molecules by the gel. In addition, this enhanced uptake is due not only to the SS in the low molecular-weight $P_{t=20}$ fraction but also to the SS in the high molecular-weight $P_{t=10}$ fraction (it should be noted that there is a difference in $P_{t=20}$ before and after shaking with the gels, although this is difficult to discern).

Previous studies have demonstrated that Con A has the ability to bind sugar molecules or sugar residues in polysaccharides.[7] This biochemical affinity could serve as an attractive force between the Con A inside the gel and the SS outside and thereby facilitate the pore diffusion of SS molecules to enhance the SS uptake by the gel. However, an expansion of the pores within the gel due to the immobilization of Con A via physical entrapment should

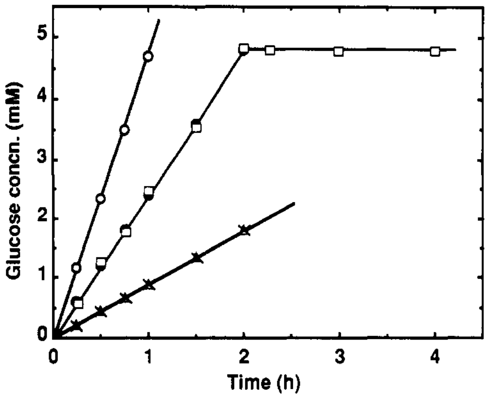

Figure 2. Glucose formation from SS as the substrate by native glucoamylase (○) and different PVA gels with immobilized glucoamylase: (△) IG; (●,□) CIGC; (×) CIGH. Total protein content for CIGC, (●) 507 and (□) 501 μg/g of gel; substrate concentration, 0.25% (w/v); pH 5.0 (50 mM citrate buffer); and 37 °C. The relative activities for each immobilized enzyme are 19.3% (IG), 19.0% (CIGH), and 51.2% (CIGC), with the rate of glucose formation by the native enzyme taken as 100% activity.

enhance the diffusion of the SS in the high molecular-weight $P_{t=10}$ fraction. To clarify the role of Con A in the pore diffusion of SS, a control experiment was carried out using a gel sample (IH in Table I) into which HSA without any binding affinity toward sugar molecules was immobilized instead of Con A. There was no marked difference in the GPC curves obtained with IH and the original PVA gel (see Figure 1). As a result, we can say that the Con A within the gel facilitates the pore diffusion of SS as a polymeric solute via its biochemical affinity and therefore enhances the uptake of SS.

Hydrolysis of Starch by Gel-Entrapped Enzymes with and without Con A. A useful application of the present Con A/gel system would be the prevention of substrate diffusion limitations in immobilized enzyme reactions, particularly in reactions involving polymeric substrates. Here, we investigated the glucoamylase-catalyzed hydrolysis of SS as the polymer substrate using the three immobilized preparations shown in Table I. This investigation is also a good tool for confirming the conclusion reached in the previous section. The enzyme used here is known to hydrolyze principally the α-1,4 linkage of the nonreducing end of the starch molecule.[14]

Figure 2 shows the time courses for glucose formation by the gels with immobilized glucoamylase (IG), coimmobilized glucoamylase and Con A (CIGC), and coimmobilized glucoamylase and HSA (CIGH), together with the results for the native enzyme. A drastic fall in enzymatic activity was observed for both IG and CIGH without Con A, that is, ca. 19% of the native enzyme activity. In contrast, CIGC with Con A exhibited considerably restored enzyme activity—51% that of the native enzyme. This observed effect of Con A is not due to the leakage of the enzyme from the gel during the measurement of activity, because it was found that the formation of glucose terminated when the CIGC was separated from a reaction mixture after the enzymatic hydrolysis was allowed to continue for 2 h.

The pH-activity profiles of three immobilized enzymes were studied and compared with that of the native enzyme (Figure 3). There was no distinguishable difference in the pH-activity curves between the immobilized and native enzymes, when the relative activity was calculated on the basis of the formation rate of glucose from SS by each of the enzyme samples at pH 5. This indicates that the restoration of activity observed in the CIGC system was not due to a shifting of the optimal pH caused by immobilization. Therefore, the Con A-induced facilitated pore diffusion of SS could serve to enhance the activity of gel-entrapped glucoamylase.

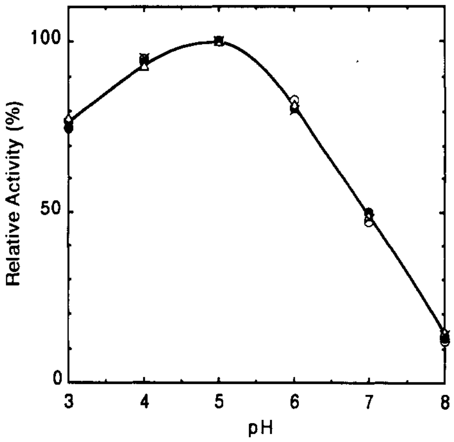

Figure 3. pH-activity curves of native and PVA gel-immobilized glucoamylase. Symbols for each plot correspond to those in Figure 2. The reaction conditions are the same as those in Figure 2 except for the pH. The relative activity is expressed, taking the reaction rate at pH 5.0 for each enzyme sample to represent 100% activity.

Kinetic Parameters of Gel-Entrapped Enzymes with and without Con A. In order to discuss the role of the Con A-induced facilitated pore diffusion of SS in the enzyme reaction in detail, kinetic data obtained at pH 5 and under different SS concentrations were examined according to the Michaelis–Menten approach. By examining the maximum velocity (V_{max}), which varies depending upon the enzyme concentration in an assay system, we can learn about the amount of effectively immobilized enzyme molecules[15] and also the interaction and/or reaction of enzymes with the supporting materials or chemicals used for immobilization.[16] From a comparison of the Michaelis–Menten constant (K_m) of the native enzyme with the apparent K_m of the immobilized enzymes, we can discuss the effects of limited substrate diffusion on immobilized enzyme activities. This is because changes in the concentration of substrates within the supporting matrices bring about an increase or a decrease in K_m through an alteration in the affinity of the enzyme toward the substrate.[4,6,15,17,18]

Figure 4 shows the Lineweaver–Burk plots for evaluating the values of V_{max} and K_m. The V_{max} values (0.10 ± 0.01 mM/min), obtained from the intercepts on the Y-axis of each straight line in Figure 4, are independent of the presence or absence of Con A within the gel. This indicates that the physical entrapment of glucoamylase with PVA gel does not cause a loss in active enzyme molecules upon immobilization. In addition, the complexation of the enzyme with Con A (see ref 10) has no influence on enzyme activity. As a result, glucoamylase molecules existing within all regions of the PVA gel seem to exhibit an activity toward SS as the polymeric substrate which is the same as that of the native enzyme.

The K_m values obtained from the intercept on the X-axis of the straight lines in Figure 4 were 0.02% (w/v) for the native enzyme, 0.34% (w/v) for CIGC, and 1.4% (w/v) for IG. A large increase in the K_m value is observed when entrapping the enzyme with PVA gel. This means a decrease in the substrate concentration within the gel phase which is due to the great resistance to SS diffusion through the gel porosity. In contrast, the coimmobilization of Con A with the enzyme made a considerable contribution to reducing the K_m value. It can thus be said that Con A in the gel phase enhances the SS concentration in the

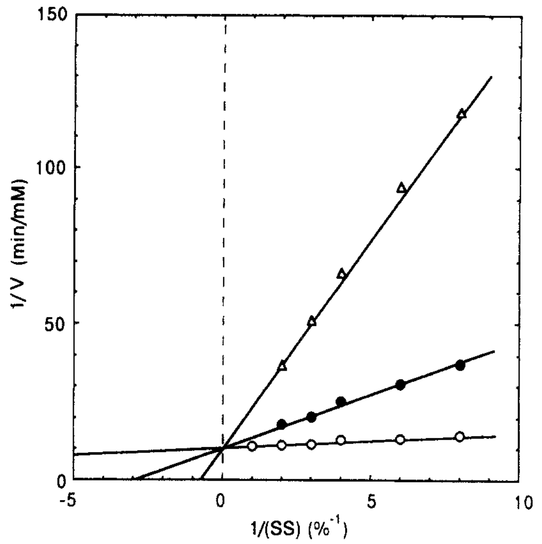

Figure 4. Lineweaver–Burk plots of native and PVA gel-immobilized glucoamylase. Symbols for each plot correspond to those in Figure 2. The reaction conditions are the same as those in Figure 2, except for the use of substrate solutions ranging in concentration from 0.125 to 1% (w/v).

immediate vicinity of the entrapped enzyme molecules. This result agrees with the conclusion from Figure 1, which showed that the lectin is capable of facilitating the pore diffusion of the polymeric solute from the aqueous to the gel phase.

Conclusion

The immobilization of Con A in PVA gel via physical entrapment makes it possible for SS to act to facilitate its pore diffusion as a polysaccharide. This contributes to an increase in enzyme activity when the glucoamylase entrapped within the Con A/gel system is subjected to the hydrolysis of SS as the polymeric substrate. It seems that the binding affinity of Con A toward SS creates an attractive force between the molecules of both substances and plays an important role in the facilitated diffusion of SS from the outside to the inside of the gel. The concept introduced here, however, should be applicable in general, and various types of biochemical or chemical binding affinities, such as antibody-antigen or receptor-substrate interactions, could be used to construct hydrogel systems that function to promote the diffusion of polymeric solutes.

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