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PREVENTION OF LIMITING SUBSTRATE DIFFUSION IN AN IMMOBILIZED ENZYME REACTION SYSTEM: LECTIN-INDUCED ACTIVATION OF GEL-ENTRAPPED β-D-GALACTOSIDASE

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SUMMARY

Escherichia coli β-D-galactosidase (EC 3.2.1.23) was entrapped in polyion complex-stabilized alginate gel beads together with a lectin from *Ricinus communis* (RCA_1 lectin). The rate of entrapped enzyme-catalyzed hydrolysis of O-nitrophenyl-β-D-galactoside dramatically increased with an increase in lectin content, and at the maximum level of lectin content the entrapped enzyme activity exceeded the native enzyme activity. A rapid decrease in the apparent K_m was observed while increasing the lectin content, whereas the V_{max} value varied insignificantly.

INTRODUCTION

Immobilized enzymes are generally subjected to external and internal diffusion limitation by substrates. In particular, this becomes problematic when enzymes are entrapped in the gel phase (e.g., Srere *et al.*, 1973). A novel approach to resolve this diffusion problem would involve the simulation of biological transport systems in which small neutral molecules penetrate membranes by facilitated diffusion which differs from usual simple diffusion.

It is well known that a mobile carrier, often a protein with high affinity for the molecule to be transported, plays an important role in the facilitated diffusion process (Metzler, 1977). This prompted us to employ a material having the ability to carry substrates in constructing gel-entrapped enzyme systems to facilitate the substrate diffusion. A castor bean lectin, RCA_1, was chosen in the present immobilization study

of β-D-galactosidase, because of its high binding affinity for β-lactose (Nicolson *et al.*, 1974). This communication describes that a marked activation effect was observed in the enzymatic hydrolysis of O-nitrophenyl-β-D-galactoside (ONPG) as a model substrate of β-lactose, when utilizing the enzyme coimmobilized with the lectin in calcium alginate gel beads stabilized by a polyelectrolyte complex consisting of potassium poly(vinyl alcohol) sulfate (KPVS) and trimethylammonium glycol chitosan (TGCI).

MATERIALS AND METHODS

RCA_1 lectin was commercially obtained from Seikagaku Kogyo Co. Ltd., Japan. β-D-Galactosidase was purchased from Worthington Company, USA. ONPG was obtained from Wako Pure Chemical Co. Ltd., Japan. Sodium alginate (SA), KPVS, and TGCI were the same polymers used in our previous studies on cell and enzyme immobilization (Kokufuta *et al.*, 1987 and 1988). All other reagents were of analytical grade.

The enzyme immobilization was carried out according to our previous method (Kokufuta *et al.*, 1988): An aqueous 2 % SA solution (24 ml) containing 29.2 mg KPVS, 30 μg enzyme, and 0 to 15 mg lectin was added dropwise to a gently stirred 100 mM $CaCl_2$ solution including 112.5 mg TGCI. The resultant gel beads (diameter = 4.5 ± 0.04 mm) were sufficiently washed with distilled water, and then equilibrated in 0.2 M Tris-HCl buffer solutions (pH 6.5 - 9) containing both 0.1 M NaCl and 0.008 M KCl.

The activities of the entrapped and native enzymes were estimated from kinetic studies of the hydrolytic reaction of ONPG at 30 °C in the buffer solution described above. Preliminary experiments revealed that this buffer solution was appropriate not only to avoid an electrostatic effect of matrix-bound charged groups, but also to prevent activation or inactivation of β-D-galactosidase caused by a slight change in the concentrations of alkaline ions in the medium (Wallenfels and Weil,1972). The assay system of the entrapped enzyme consisted of 6 ml of the substrate solution plus 4 ml of the gel beads containing 5 μg of the enzyme, whereas the native enzyme was assayed in 10 ml of the substrate solution including the same amount of the enzyme as used in the immobilized preparation. The substrate solutions in both assay systems included 0.08 to 4.0 mM of ONPG. The concentration of O-nitrophenol resulting from the hydrolysis of the substrate was determined colorimetrically at 420 nm.

The distribution of the substrate between the gel and water phases was studied: The gel beads (5 ml; lectin content = 0 - 0.625 mg/ml gel) were shaken at 30 °C in 5 ml of the substrate solution (initial ONPG concn. = 0.055 mM; pH 7.25) until equilibrium was established, then the equilibrium concentration of ONPG in the aqueous phase was determined spectrophotometrically at 280 nm. The amount of ONPG incorporated into the gel beads was calculated from a difference in the ONPG concentration between the initial and equilibrium states.

RESULTS AND DISCUSSION

Leakage of the entrapped enzyme and lectin from the polyion complex-stabilized gel beads was tested prior to investigating the immobilized enzyme activity. It was found that both proteins were completely held in the gel support for at least 3 days under the conditions used in the activity measurement or the distribution experiment.

Figure 1 depicts the effect of the lectin content ($[L]_g$) on the immobilized enzyme activity. The dashed line indicates 100 % relative activity referring to the rate of ONPG hydrolysis by the native enzyme. The error symbols represent the standard deviations determined on the five repeated measurements. The activity of β-D-galactosidase entrapped in the gel beads in the absence of the lectin was about 35 % of the native enzyme activity. However, the presence of the lectin in the gel led to a dramatic increase in activity, and at the level of $[L]_g$ = 0.625 mg/ml gel the immobilized enzyme activity exceeded the native enzyme activity by approximately 10 %.

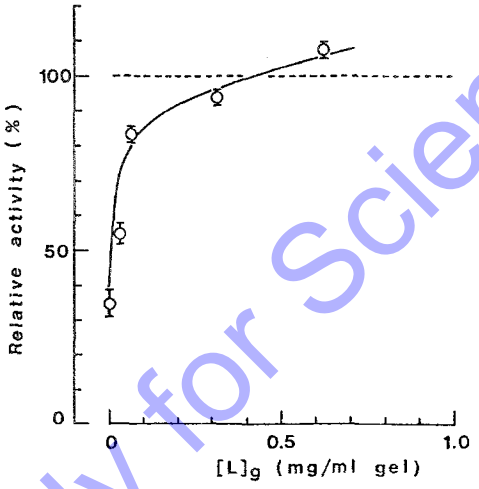

Fig. 1. Change in activity of polyion complex-stabilized alginate gel-entrapped β-D-galactosidase with $[L]_g$. Enzyme content, 0.5 μg/ml gel; substrate concn., 4.0 mM; pH 7.25.

There was no distinguishable difference in the pH-activity profiles between the immobilized and native enzymes in the pH 6.5 to 9 range. This is because the immobilized enzyme activity was assayed with care to eliminate the influence of ionizable groups bound to the supporting matrix (see experimental section). Thus, it is not necessary to take into account change in activity due to a shift of optimal pH.

It was observed that activity failed to increase in the immobilized system, when the RCA_1 lectin was replaced by the other type of plant lectin, i.e., concanavalin A (from jack bean) having a high binding affinity toward α-D-mannopyranose or α-D-glucopyranose (Kornfeld and Ferris, 1975). This indicates that the binding specificity of the RCA_1 lectin for ONPG is a significant factor in the increased activity of the immobilized preparation. From careful gel-filtration chromatographic studies with a Toyo Pearl HW-60F column, it was found that no complex formation occurred between the lectin and enzyme. This suggests that the interaction between both proteins is negligible in the lectin-activated immobilized enzyme reaction. As a result, it is reasonable to assume that the lectin in the gel phase strongly attracts the substrate from the aqueous phase and this leads to an increase in the apparent substrate concentration of the gel phase.

In order to confirm this assumption, the Michaelis-Menten constants were estimated by analyzing kinetic data obtained under different concentrations of the substrate, using the Lineweaver-Burk method. The variation in the obtained V_{max} values (0.70 ± 0.05 μM/min) was found to be independent of $[L]_g$ and within the limit of the error in repeated activity measurement. In contrast, the apparent K_m value rapidly decreased with increasing $[L]_g$ (see Figure 2). Especially, the K_m value of the immobilized enzyme at $[L]_g$ = 0.625 mg/ml gel was rather lower than that of the native enzyme indicated by the dashed line. These results strongly suggest that the lectin in the gel phase plays an important role in enhancing the

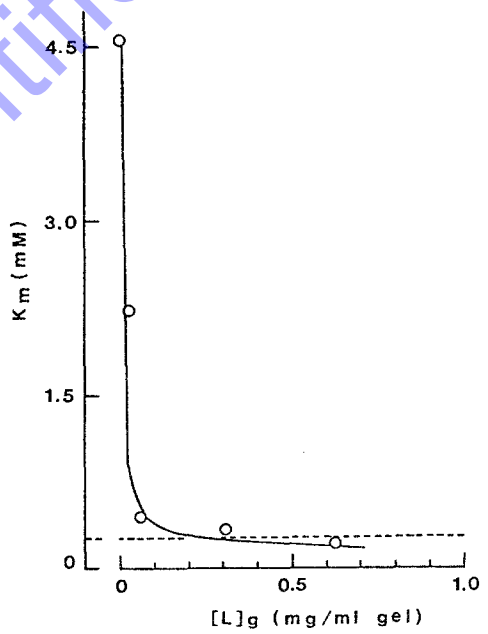

Fig. 2. Change in K_m with $[L]_g$. Enzyme content, 0.5 μg/ml gel; substrate concn., 4.0 mM; pH 7.25.

substrate concentration in the immediate vicinity of the immobilized enzyme molecules. In other words, the lectin is capable of facilitating the substrate diffusion from the aqueous to the gel phase.

Further information about the role of the lectin could be obtained from examining the partition of the substrate between the gel and water phases. Figure 3 shows the effect of $[L]_g$ on the partition coefficient (P) expressed as the ratio of the equilibrium concentrations (mM) of ONPG in the gel and aqueous phases. It was found that the value of P increased linearly with $[L]_g$. In our partition experiments, the initial ONPG concentration was kept constant; therefore, the obtained linear relationship suggests very high binding affinity of the lectin with ONPG. This high affinity can be expected to act as a driving force for concentrating ONPG in the gel phase.

The observed lectin-induced activation of the gel entrapped β-D-galactosidase may be interpreted as shown in Figure 4: The lectin (L) in the gel beads attracts the substrate (S) from the bulk water to form a complex (L-S). When the resulting L-S

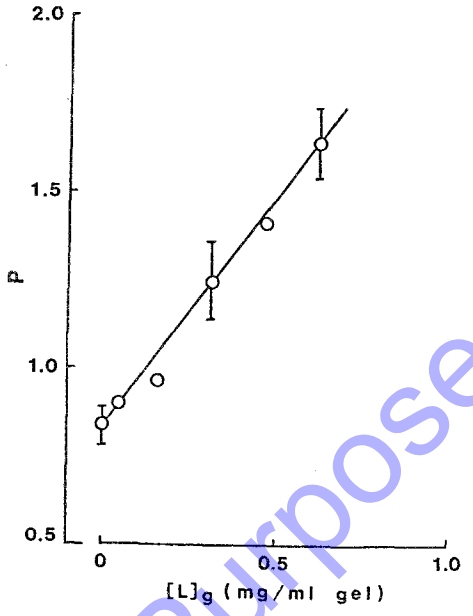

Fig. 3. Change in P with $[L]_g$ for the enzyme-free gel beads. Initial substrate concn., 0.055 mM; pH 7.25.

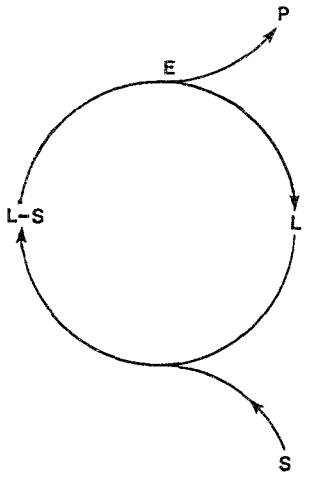

Fig. 4. Schematic representation for understanding the lectin-induced activation of the entrapped enzyme.

complex is hydrolyzed by the catalytic action of the enzyme (E) to yield the product (P), the regenerated L again forms the L-S complex and the process is repeated. Even though diffusion rates of L-S and L with high molecular weights within the gel phase are small, the rate of the enzyme reaction can increase with increasing $[L]_g$ to easily bring the L-S complex into contact with E. In addition, the high binding affinity of L with S results in a large difference in the concentration of S between the outside and the inside of the gel bead, because of a slight amount of S in the free form within the gel phase. These features appear to be similar to the carrier-mediated facilitated diffusion which is a common transport process in biological systems.

In conclusion, it is our view that the present method for constructing a gel-entrapped enzyme system, which was designed by simulating a biological transport system, assists in developing solutions for the problem of diffusion limitation in immobilized enzyme reactions.

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