

## Sugar-responsible Initiation–Cessation Control of Enzyme Release from Gel Matrix by use of an Agarose/Concanavalin A System

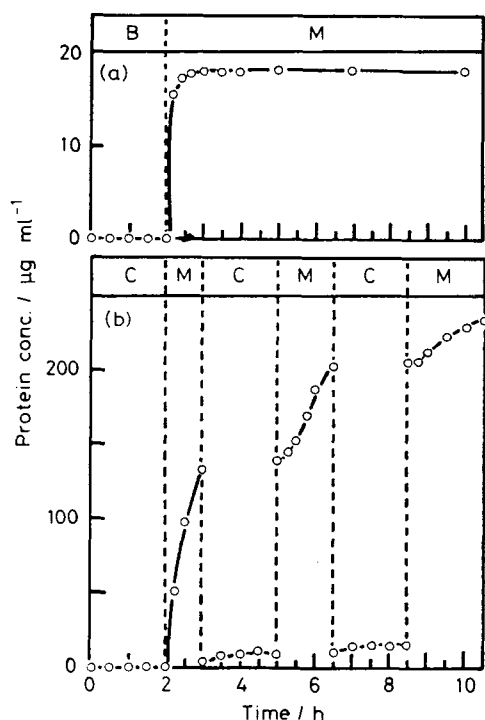
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The diffusional release of *exo*-1,4- $\alpha$ -D-glucosidase from agarose gel stops when Concanavalin A binds to the gel, but its removal from the gel by D-mannose leads to rapid release; the reversible initiation–cessation control can be achieved in both batchwise and column operations by the alternate use of D-mannose and Concanavalin A.

Recently, the regulation of diffusional solute release from polymeric matrices by an external stimulation has drawn much attention with regard to developing a drug delivery device. Several attempts have been undertaken to control the release and the release rate using an appropriate externally applied signal; *e.g.*, glucose-stimulated release of glycosylated insulin from the membrane which separates its complex with Concan-

avalin A (Con A),<sup>1</sup> amino compound-sensitive release of methyl orange from an amphiphilic polymer membrane,<sup>2</sup> electrochemically regulated release of glutamate from a conductive polymer electrode,<sup>3</sup> and thermally controlled release of organic substances (indomethacin and vitamin B<sub>12</sub>) from a thermosensitive polymer gel.<sup>4</sup> In this report, we describe a novel enzyme-delivery system in which the initia-

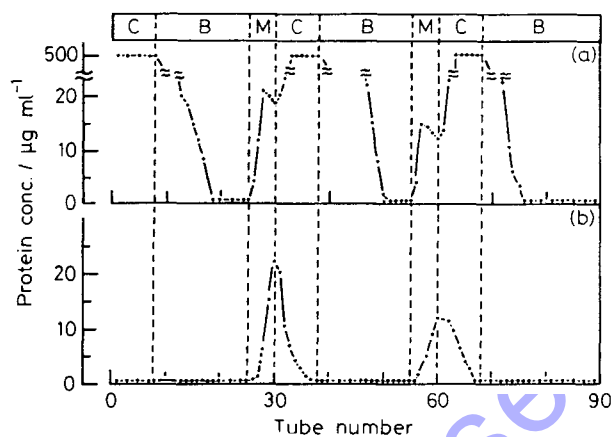


**Figure 1.** Time courses of Con A dissociation (a) and EG release (b) in the batchwise operations carried out at 25 °C, using 50 mM citrate buffer (B), 100 mM D-mannose solution (M), and 500 µg/ml Con A solution (C). The sample sizes used for determining the protein concentrations were 60 µl for Con A by the dye-binding assay method and 20 µl for EG by the enzymatic activity measurement.

tion and cessation of the diffusional release of *exo*-1,4-D-glucosidase (EG) through the pores of agarose gel (AG) can be reversibly controlled. This is because the binding affinity of Con A towards AG is dramatically altered in response to the presence and absence of D-mannose (M) and also because the bound Con A-induced steric hindrance effect strongly inhibits the diffusional release of the enzyme through the gel porosity.

The physical properties of both proteins used are: molecular weight (*M*),  $1.1 \times 10^5$  (EG) and  $5.2 \times 10^4$  (Con A as a dimer at pH 5.0); isoelectric point, 4.2 (EG) and  $5 \pm 0.5$  (Con A). The cubic and beaded AG preparations were used for studying EG release in batchwise and column operations, respectively. Citrate buffer (50 mM, pH 5.0) was employed in all the experiments. The gel cubes (each side 5 mm) were obtained by cooling 10 ml of a hot solution (48 °C) containing agarose (150 mg) and EG (51.4 mg) in a plastic container, followed by cutting into exactly size-fixed segments with a slicer. The gel beads (mean diameter 2 mm) were formed by dropping a thermostated, 1.5% agarose solution (48 °C and about 50 ml) into a precooled and gently stirred 3:2 mixture (500 ml) of light petroleum and dichloromethane through a capillary pipette. The resulting gel beads were then washed thoroughly with the buffer solution and subjected to the EG loading procedure. The enzyme was loaded by stirring 20 ml of the gel beads in the buffer (10 ml) including 5 mg of EG for 12 h in a refrigerator (amount of EG loaded = 167 µg/ml gel).

At present the literature<sup>5</sup> contains many references to the sugar binding affinities towards Con A. However, there are no available investigations dealing with the AG/Con A system. Thus, we first studied the effect of M on the binding affinity of Con A to AG. The enzyme-free AG cubes (1 ml), to which 362



**Figure 2.** Changes in the concentrations of Con A (a) and EG (b) in the effluent fraction during the column operation. Each of the abbreviations used for indicating the eluting agents corresponds to Figure 1. The eluting agent was passed through the column at a constant flow rate (0.1 ml/min), and the effluent fraction was received in a test tube (5 ml per tube). The concentration of EG was determined by measuring its enzymatic activity, while that of Con A was estimated by subtracting the EG concentration from the total protein concentration determined by the dye-binding assay method.

µg of Con A was previously attached, were used in the experiments. The staining of the gel sample using Coomassie Brilliant Blue G-250 (CBB) showed that the protein combined to form a thick, opaque layer around the surface of the gel matrix. The Con A-attached gel cubes were mildly stirred in the buffer solution followed by the subsequent sugar solution (19 ml each). The amount of Con A dissociated from the gel into the aqueous phase was determined colourimetrically, according to the dye-binding assay with CBB.<sup>6</sup> As shown in Figure 1(a), the Con A bound to AG did not dissociate in the buffer solution, but it did dissociate in the sugar solution. Assuming that the binding of Con A to AG is pictured as  $AG + Con A = AG-Con A$  (equation 1), this result suggests that in the absence of M the equilibrium constant becomes very large. In the presence of M having very high binding affinity towards Con A,<sup>5</sup> however, the equilibrium constant dramatically decreases, since the following exchange reaction becomes a dominant factor:  $AG-Con A + M = M-Con A + AG$  (equation 2). As a result, this favourable feature prompted us to apply it to the construction of a sugar-stimulated protein delivery system.

Figure 1(b) shows typical time course of EG release in the batchwise operation. This was performed by mildly stirring the enzyme-loaded gel cubes (1 ml) in both Con A and M solutions (19 ml each) alternately. The released amount of EG was monitored by investigating its hydrolytic activity towards soluble starch as a substrate.<sup>7</sup> The enzyme release is sufficiently depressed in the Con A solution, whereas replacing it by the sugar solution leads to the prompt release. Such an initiation-cessation control was reversibly repeated until 92% of the loaded enzyme was released. Therefore, it can be said that the enzyme molecules diffuse without difficulty through the gel porosity even if they are present in any site within the gel phase.

The initiation and cessation of EG release can be regulated in the continuous operation (at 25 °C), using a column (10 mm inside diameter; 400 mm height) packed with 5 ml of the enzyme-loaded gel beads. The same Con A and sugar solutions as used in the batchwise operation were also

employed as an eluting agent. As can be seen from Figure 2, when passing the M solution through the column, the Con A bound to AG is rapidly excluded, accompanying the EG release. However, such an enzyme release can be depressed by the replacement of the eluting agent by the Con A solution. This depressed enzyme release was maintained even when a large amount of buffer solution was then passed through the column.

From careful gel-filtration chromatographic studies, it has been found that no complex formation occurred between EG and Con A. In addition, the shrinkage of AG was not observed when Con A was bound to the gel. The depression of EG release from AG can thus be explained on the basis of a bound Con A-induced steric hindrance effect on the diffusion of the enzyme through the gel porosity. The removal of the bound Con A from the gel using the sugar (equation 2) seems to eliminate such a hindrance effect and thus enable the enzyme diffusion. Therefore, the principle of the present system is distinct from those of the previous systems based on signal-dependent alterations in the stability of Con A complexed with the solute to be released<sup>1</sup> and also in the swelling degree of polymer membrane<sup>2</sup> or gel.<sup>4</sup>

The sugar-responsive initiation-cessation control of enzyme release was also useful for other enzymes having different molecular weights; for example, ribose isomerase ( $M 1.6 \pm 0.6 \times 10^4$ ) and tryptophanase ( $M 2.2 \times 10^5$ ).

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