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Study on the Release of Invertase from Enzymatically Degradable Dextran Hydrogels

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ABSTRACT

Chemically crosslinked dextran hydrogels were prepared for application in the controlled delivery of bioactive proteins. Dextran was functionalized by reacting with glycidyl acrylate to introduce reactive double bonds. Upon exposure to γ-irradiation the functionalized dextran formed a crosslinked gel which could be degraded by dextranase. The effect of dextranase-induced degradation on the swelling kinetics of the prepared hydrogels was examined. Enzymatic degradation of the gels became slower as the γ-irradiation dose increased for the formation of the gels. The dextran hydrogels were examined as a potential delivery system for proteins by using invertase as a model protein. Invertase was incorporated into the hydrogel by mixing it with the purified, functionalized dextran before exposure to γ-irradiation. The effect of γ-irradiation on the bioactivity of the incorporated invertase was determined. The γ-irradiation did not change the bioactivity of the incorporated invertase as long as the total γ-irradiation dose was limited below 0·4 Mrad. The release study showed that the release of invertase from the dextran gel was controlled by dextranase-induced degradation rather than diffusion through the dextran network. The release study also showed that the invertase release was pulsatile. Parameters such as the degree of functionalization, dextran molecular weight, and γ-irradiation dose can be adjusted to prepare delivery systems which meet the desired degradation kinetics and protein release profiles.

INTRODUCTION

Not many topics in the area of drug delivery have provoked as much debate and interest as that of the delivery of bioactive peptides and

proteins. Despite much effort, the development of suitable delivery systems remains as one of the bottlenecks for the successful use of peptide and protein drugs. For the delivery systems to be useful, problems associated with the loading, stability and release of the protein drugs need to be overcome. Various approaches have been tried to overcome such problems.[1–5] Of the many approaches, biodegradable hydrogels present unique advantages in terms of controlling the release profile of high molecular weight drugs and removal of the delivery system after its use *in vivo*.[6] Unlike the diffusion-controlled release through non-degradable polymer matrices, the use of biodegradable hydrogel provides an alternative release mechanism which is predominantly governed by the degradation of the network. Thus, the release of protein drugs from such systems can be adjusted by controlling the degradation kinetics of the hydrogels.

Traditionally, loading of peptide and protein drugs in the hydrogels has been carried out by one of the following two methods: preparation of hydrogels in the presence of drugs; and swelling of dried hydrogels in the drug solution. The first method is undesirable due to the presence of residual initiators, monomers and crosslinkers which tend to be toxic. In addition, the increase in temperature during polymerization may have an adverse effect on the bioactivity of the protein drugs being incorporated. The loading of protein drugs by equilibrating the hydrogels in the drug-containing solution usually results in poor loading. This is mainly because diffusion of protein drugs into the hydrogel network is very slow and limited by the pore size of the network. New, simple approaches for the protein loading would be highly beneficial in the development of useful protein delivery systems.

In our previous study, we prepared gelatin hydrogels.[7,8] Gelatin was functionalized using glycidyl acrylate to introduce pendant double bonds. The functionalized gelatin molecules performed as macromonomers as well as crosslinkers by virtue of double bonds introduced to their structure. These macromonomers were subsequently crosslinked by γ-irradiation. This approach allowed preparation of hydrogels without the use of externally added crosslinkers. In addition, the functionalized biopolymers could be purified prior to gel formation, and thus, the prepared gels did not have to be purified further. The gelatin hydrogels are good candidates for the delivery of non-protein drugs such as oligonucleotides which are not degraded by proteases.

In this study we were interested in developing biodegradable protein delivery systems. For this reason, we decided to synthesize hydrogels made of polysaccharides. We chose dextran and invertase as a model polysaccharide and a model protein drug, respectively. Invertase was

preferred over others because of its easily measurable bioactivity and its high molecular weight (270 000 daltons) which limits the diffusional release from the gel.

MATERIALS AND METHODS

Preparation of chemically crosslinked dextran hydrogels

Dextrans (M.W. of 480 000 and 2 000 000, Sigma) were dissolved in phosphate-buffered saline solution (PBS, pH 7·2) to obtain the final concentration of 50 mg/ml. For the functionalization of dextran molecules, 0·8 ml of glycidyl acrylate (Aldrich) was added to 20 ml of the dextran solution and the reaction was allowed to proceed for 2 days at room temperature under constant stirring. After 2 days, 4 ml of 20% (w/v) glycine solution was added to stop the functionalization reaction and stirring continued for another 30 min. The whole solution was then dialyzed against PBS (pH 7·2) for 2 days with 10 changes of the buffer solution to purify the functionalized dextran. Functionalization of proteins[9–11] and other polysaccharides[2,12,13] based on the similar aproach has been reported in the literature. The content of acrylic groups in the functionalized dextran was determined spectrophotometrically by measuring the decrease in absorbance at 480 nm in methanol–water (80:20) solution of bromine (0·2% v/v).[2,14] Acrylamide was used as a standard for this assay.

The hydrogels were prepared in graduated polystyrene test tubes (Becton Dickinson Labware, dimensions 17 mm × 100 mm). Functionalized dextran was transferred to these test tubes and γ-irradiated for 4 h, 6 h or 8 h. Prior to irradiation, the samples were degassed and purged with nitrogen. The cylindrical gels were removed from the test tubes, washed with distilled water to remove any water-soluble components, and cut into disks. These disks were then air dried for 24 h and oven dried for 12 h at 60°C. The dimensions of the dry gel disks were 12 mm × 2 mm.

Swelling and degradation kinetics

The effects of γ-irradiation dose on hydrogel swelling and dextranase-induced degradation were determined by examining the swelling kinetics of the gels in the absence and presence of dextranase. For the swelling study, pH 6 phosphate-buffer saline solution was used. The swelling ratio, Q, was calculated from the following equation:

$$Q = W^*/W$$

where W^* and W are the weights of the swollen and dry gels, respectively.[11,15] W is the weight of the dry gel determined before the swelling experiment. It is noted that for measuring the true swelling ratio of degradable gels, W should be the weight of the dry gel determined at the time point of each sampling rather than at the beginning of the experiment. This, however, requires a very large number of samples. Since our goal was simply to monitor and compare the changes in the hydrogel size, we used the value determined in the beginning of the experiment for both non-degradable and degradable gels.

Release of invertase from dextran hydrogels

Our preliminary study showed that the dextan gels prepared by a total dose of 0·64 Mrad (which was equivalent to 8 h of γ-irradiation time in our study) showed better mechanical integrity than those prepared by a lower γ-irradiation dose. For this reason, we incorporated invertase into this set of hydrogels. Since loading of invertase into the hydrogels involved its exposure to γ-irradiation, the effect of γ-irradiation on its bioactivity was examined. Invertase in PBS was γ-irradiated from 4 h (0·32 Mrad) to 32 h (2·6 Mrad). Because invertase breaks down sucrose into glucose and fructose, its bioactivity could be easily measured by monitoring the glucose release.

To examine the release of invertase from dextran gels, invertase was incorporated into the hydrogels by mixing it with functionalized dextran (M.W. 480 000) to obtain the final concentration of 45 units/ml. The solution was γ-irradiated for 8 h (0·64 Mrad) to form a gel. Figure 1 shows the schematic description of hydrogel formation in the presence of invertase. The invertase-containing hydrogels were dried for 24 h at room temperature and for another 24 h at 37°C. The release of invertase from the gels was examined by incubation of the dry gel disks in 25 ml of PBS (pH 6) in the absence and presence of dextranase (0·05 units/ml) at 37°C. PBS at pH 6 was used since dextranase is able to break down dextran only at pH 6 and not at pH 7·4. The weights of the dry gels ranged between 0·028 g and 0·035 g. The dimensions of the dry gel disks used for the study were 12 mm × 2 mm. Each gel contained about 45 units of invertase. The gel disks were placed on a wire mesh, immersed half-way through the buffer in 50 ml glass beakers by means of a holder. These were maintained at 37°C using a circulating water bath and were covered using a polymer sheet on top to minimize the loss of buffer by evaporation. The buffer solutions in the glass containers were stirred using magnetic stirring bars throughout the

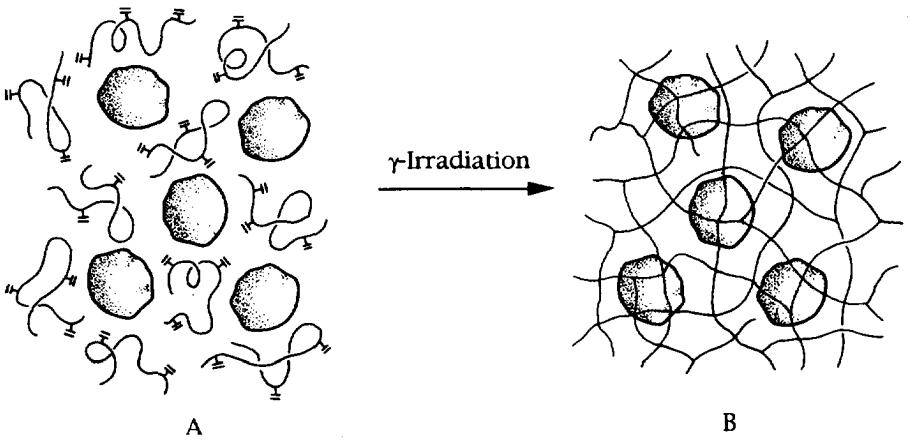

Fig. 1. Schematic description of invertase incorporation into hydrogels. A: Invertase (circular objects) was mixed with purified, functionalized dextran (random coils with introduced double bonds). B: The solution forms a hydrogel containing invertase upon exposure to γ-irradiation.

release experiments. At timed intervals, 2 ml aliquot was removed for the invertase assay, and 2 ml of fresh PBS, either with or without dextranase (0·05 units/ml), was added to the medium. The invertase activity was determined by mixing the aliquot with 2 ml of 0·3 M sucrose and 2 ml of pH 4·5 acetate buffer at 55°C for 1 h. The generated glucose was determined using Glucose (HK) reagent (Sigma). Prior to the invertase release study, the invertase activity was calculated after compensating for the loss in activity at the end of 8 h of γ-irradiation. The effect of irradiation intensity rather than the irradiation dose size was not determined in our study, since the Co-60 source of 0·0804 Mrad/h was the only available irradiation source for our study. The dextran gels which did not contain invertase were handled in the same manner to make sure that there were no impurities which may affect the glucose test either before or after enzymatic degradation.

RESULTS AND DISCUSSION

The degree of functionalization of dextran was found to depend on its molecular weight. One acrylic group was introduced per 1·83 glucose residues of dextran when the molecular weight of dextran was 480 000. On the other hand, the extent of functionalization was only one acrylic group per 10·9 glucose residues when the molecular weight was

2 000 000. The substantially lower degree of functionalization of the latter could be due to higher viscosity and/or chain entanglement which hindered the approach of glycidyl acrylate to the desired reaction sites.

The mechanical integrity of the hydrogels increased as the γ-irradiation dose increased up to the total dose of 0·64 Mrad (8 h of γ-irradiation) used in this study. Thus, dextran gels prepared by 0·64 Mrad of γ-irradiation were used for subsequent studies. The swelling profile of these gels was dependent on the γ-irradiation time (or the total γ-irradiation dose) and the dextran molecular weight (Fig. 2). For both molecular weights of dextran, the swelling ratio decreased as the γ-irradiation time increased from 4 h to 8 h. This is due to the higher crosslinking density at increased γ-irradiation dose. The gels prepared from 480 000 dalton dextran showed lower swelling ratios as compared to those prepared from the higher molecular weight counterpart. This was expected since the extent of functionalization was much higher with the lower molecular weight dextran as described above.

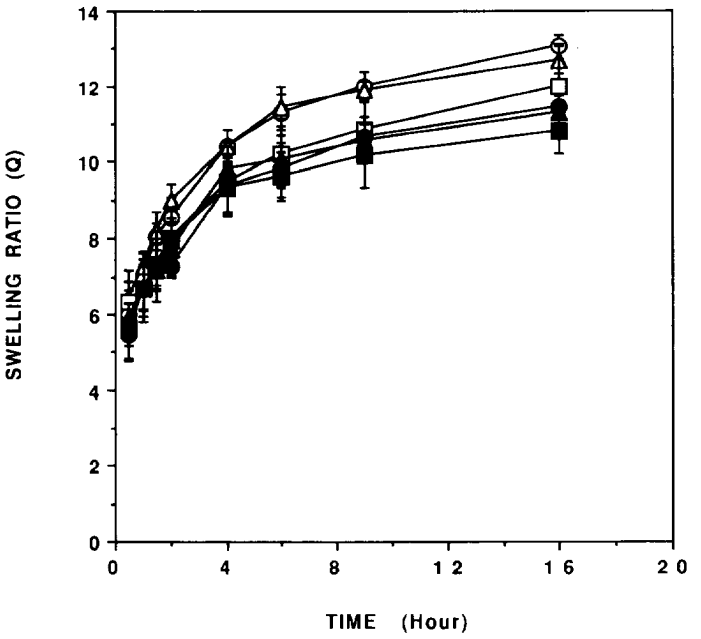

Fig. 2. Comparison of the swelling kinetics of dextran hydrogels in the absence of dextranase. The hydrogels were prepared from dextran by γ-irradiation for 4 h (○, ●), 6 h (△, ▲) and 8 h (□, ■) at the dose rate of 0·0804 Mrad/h. The concentration of dextran was 50 mg/ml and the molecular weights were 2 000 000 (open symbols) and 480 000 daltons (closed symbols) ($n = 4$).

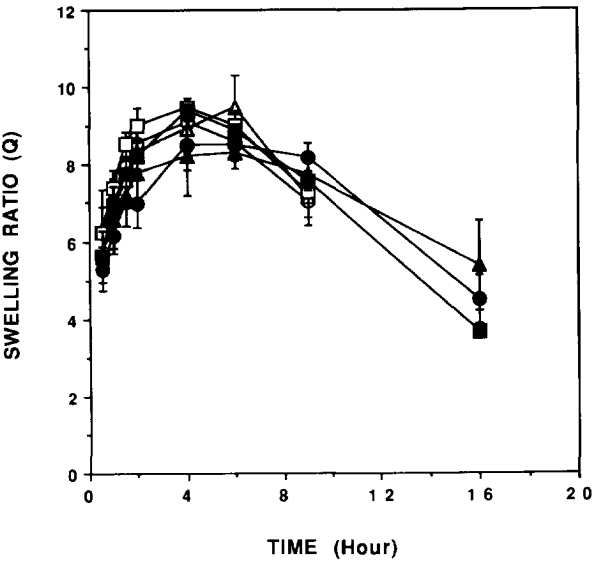

Fig. 3. Comparison of the swelling kinetics of dextran hydrogels in the presence of dextranase. The hydrogels were prepared from dextran by γ-irradiation for 4 h (○, ●), 6 h (△, ▲) and 8 h (□, ■) at the dose rate of 0·0804 Mrad/h. The concentration of dextran was 50 mg/ml and the molecular weights were 2 000 000 (open symbols) and 480 000 daltons (closed symbols) ($n = 4$).

The swelling of dextran hydrogels in the presence of dextranase is shown in Fig. 3. The effect of γ-irradiation time on the swelling ratio was not as clear as seen in Fig. 2. After 16 h in the presence of dextranase, the gels degraded to such an extent that they behaved like a viscous solution and it was difficult to separate them from the solution to measure the swelling ratio. The gels completely dissolved after 30 h as determined by visual observation. The swelling profile of degrading hydrogels usually shows a transient maximum swelling ratio as shown in Fig. 3. The gels prepared from 2 000 000 dalton dextran degraded faster than the 480 000 dalton dextran gels. This higher susceptibility to enzymatic degradation is consistent with the swelling data in Fig. 2 which indicate the loosely crosslinked network with 2 000 000 dalton dextran. The maximum swelling ratios observed at hour 4 in Fig. 3 are smaller than those observed at the same time point in the absence of dextranase in Fig. 2. This indicates that the degradation of the dextran gels is mainly by surface-degradation.[11] It is interesting to notice that the degree of swelling and degradation rates in Figs 2 and 3 are very close for the network formed by two different dextrans despite the large difference in the degree of functionalization. This may be due to the

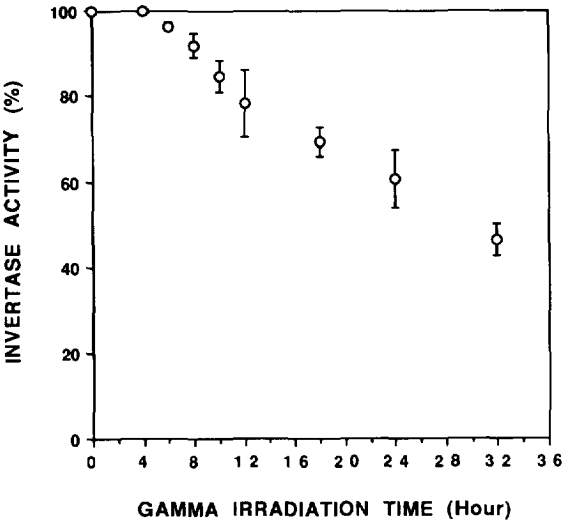

Fig. 4. Effect of γ-irradiation on the bioactivity of invertase. 45 units/ml of invertase was γ-irradiated for various time period at the dose rate of 0·0804 Mrad/h. The activity of invertase is expressed as a percentage of the native invertase activity.

different efficacy of γ-irradiation in the crosslinking reaction of different dextrans.

Since loading of invertase into the gels requires exposure of the protein to γ-irradiation, we examined the effect of γ-irradiation on the bioactivity of invertase. In Fig. 4, the bioactivity of γ-irradiated invertase is expressed as a percentage of the bioactivity of the native invertase. No decrease in bioactivity was observed when the γ-irradiation time was less than 4 h (the total dose of 0·32 Mrad). As the γ-irradiation time increased from 4 h to 32 h, there was a progressive decrease in the bioactivity of invertase. Almost 20% of the enzyme activity was lost after 12 h of γ-irradiation (total dose of 0·96 Mrad). At the end of 32 h (total dose of 2·56 Mrad), only 46% of the bioactivity was maintained. Since the hydrogel was formed by 8 h of γ-irradiation in this study, about 8% of the invertase activity was lost during loading of invertase into the gels. Since the gels can be formed by a much lower γ-irradiation dose, if parameters such as the degree of functionalization of dextran are adjusted, the decrease in the bioactivity of the protein could be reduced to negligible values. The data in Fig. 4 indicate that too much bioactivity is lost if the γ-irradiation dose is increased to 2·5 Mrad, which is the dose commonly used for sterilization.

Once we were able to load invertase into the dextran hydrogels without significant loss of its bioactivity, we examined the release of

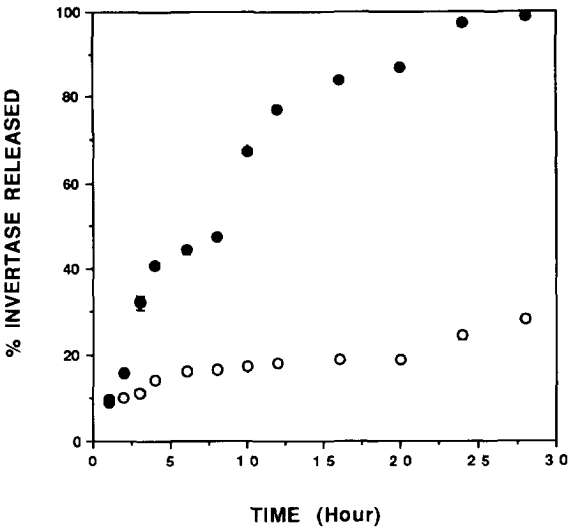

Fig. 5. Cumulative amount of invertase released from dextran gels in the absence (○) and presence (●) of dextranase. The gels were prepared from functionalized dextran of 480 000 daltons. The total γ-irradiation dose was 0·64 Mrad. The absence of an error bar indicates that the standard deviation of the data was smaller than the size of the symbol ($n = 3$).

invertase from the dextran gels in the absence and presence of dextranase (Fig. 5). In Fig. 5, the percentage of the released invertase was determined on the basis of the invertase remaining active after 8 h of γ-irradiation. In the absence of dextranase, the total amount of released invertase was less than 30% of the incorporated invertase, even after 28 h of incubation. On the other hand, in the presence of dextranase almost all of the incorporated invertase was released from the gel during the same time period. As shown in Figs 2 and 3, the swelling ratios of the gels in the absence and presence of dextranase were comparable up to 4 h. However, the amount of invertase released during the first 4 h in the presence of dextranase was significantly larger than the control values (Fig. 5). As opposed to about 15% of invertase released from the gels in the absence of dextranase, about 40% was released in the presence of dextranase during the first 4 h. It is thought that the invertase released in the absence of dextranase is mainly that which is trapped in the outer layer of the dextran gel.

The release profile of invertase in the presence of dextranase in Fig. 5 is quite interesting. The release was not monotonic. The amount of invertase released in a given time period (or the drug release rate) was not predictable. It was neither constant nor continuously decreasing. In

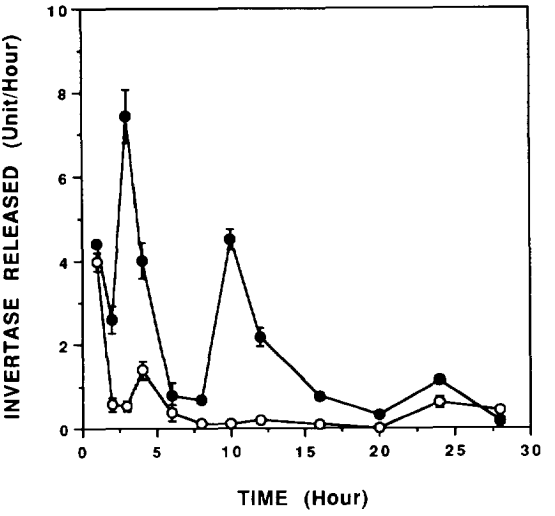

Fig. 6. The rate of invertase release from the dextran gels in the absence (○) and presence (●) of dextranase.

general, the drug release rate is expected to decrease in time. In the invertase release from the dextran gels, however, the faster and slower release rates alternated until all the incorporated invertase was released. To appreciate this unusual release profile, the amount of invertase released per unit time was plotted in Fig. 6. The rates of invertase release in the absence and the presence of dextranase were about the same in the first hour. The release rate in the absence of dextranase in the first hour was much higher than the rates observed in the later times. This suggests that the burst release in the first hour is most likely due to the release of invertase from the gel surface. Small increases in the release rate in the absence of dextranase at 4 h and 24 h time points are thought to be due to diffusion of invertase through the dextran chains of the gel. This may occur as a result of the relaxation of dextran molecules in the gel. Considering the possibility that the γ-irradiaton may not crosslink all the available acrylic groups on the dextran molecules, it is reasonable to assume that many dextran molecules are free to move in the gel and this may result in the diffusional release of invertase from the gel. In the presence of dextranase, three distinctly high release rates were observed. The small release rate followed each of the three high release rates. This clearly shows the pulsatile release of invertase from the dextran gel in the presence of dextranase. This pulsatile release may be due to the dextranase-induced surface degradation of the gel. Since invertase is a large molecule, its diffusional

release is limited. As the dextran chains are cleaved by dextranase, the pore size will be gradually increased and at some point a large amount of invertase will be released. After a burst release, the invertase release is expected to be slowed down until a sufficient number of dextran chains are cleaved again. The surface degradation of the gels also explains the progressive decrease in the peak release rate of the three pulsatile releases (at 3 h, 10 h and 24 h time points). As the size of the gel becomes smaller due to the surface degradation, the amount of invertase released from the gel also decreases. The proposed process of pulsatile release of the incorporated invertase needs to be verified by further experiments. This non-conventional pulsatile release may be useful in the delivery of certain drugs which do not have to be released at a constant rate. The degradation kinetics, and thus the release kinetics, of the dextran gels can be adjusted by controlling the experimental parameters, such as the concentration of dextran, the molecular weight of dextran, the degree of dextran functionalization, and the γ-irradiation dose. Other polysaccharides, such as hyaluronic acid and chondroitin sulfate, which are known to degrade in the human body can also be used to prepare a pulsatile delivery systems for a variety of protein drugs. This approach described here may be an alternative or an additional one to other approaches for pulsatile protein delivery.[16]

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