Mechanism and Characteristics of Protein Release from Lactitol-Based Cross-linked Hydrogel

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Lactitol-based cross-linked hydrogel was synthesized, and model proteins (α-chymotrypsin, β-lactoglobulin, bovine serum albumin (BSA), and γ-globulin) were incorporated into the cross-linked hydrogel. The larger-molecular-weight proteins have lower diffusivity (D_e) in the hydrogel. Increasing temperature accelerated the diffusion rate of proteins; however, the diffusion did not follow the Arrhenius equation at temperatures above 37 °C. The swelling ratio of the hydrogel was slightly decreased after heating for 2 h at 37 and 45 °C, and significantly reduced after 1 h at 60 °C. Therefore, diffusion of β-lactoglobulin and BSA may be decreased by hydrogel shrinking at temperature over 37 °C. The model proteins have high affinities to buffer solution compared to the hydrogel network structure, resulting in high partition coefficients ($K > 1$) which do not affect the calculation of D_e values. Incorporated protein release follows the theory of hindered diffusion.

Keywords: *Protein delivery systems; hindered diffusion; hydrogel; viscosity; swelling ratio; partition coefficient*

INTRODUCTION

Because of the high specificity of bioactive functions, proteins and peptides are produced through biotechnology in large quantities to be utilized for their functions. However, low bioavailability of the protein and peptide drugs through oral administration motivates applications of parenteral delivery (Hennink et al., 1997). Proteins and peptides are macromolecules which have various charge densities and binding sites. Therefore, their release rates from a matrix system are highly dependent on the multiple forces of interactions. They are also very sensitive to environmental conditions and may become denatured. The delivery matrix systems have to provide mild environments to the proteins and peptides to maintain their original conformations and specific activities.

Hydrogels are hydrophilic three-dimensional network gels which can absorb much more water than their own weight so as to provide ideal aqueous conditions for biocompatible applications and for environmentally sensitive bioactive materials such as proteins and peptides (Dordick et al., 1994; Park and Park, 1996; Han et al., 2000). A series of thermo-sensitive hydrogels have been produced from lactitol-based polyether polyols (LPEPs). Wilson et al. (1996) characterized the LPEP and examined the effects of reaction conditions on the characteristics of LPEPs. The LPEPs were also synthe-

sized from unpurified whey permeate and used to produce rigid polyurethane foam (Hu et al., 1997). Lin et al. (1998) synthesized and characterized the cross-linked LPEP hydrogels. Han et al. (2000) utilized the LPEP hydrogel for a controlled chemical-delivery system. The release rate of aspirin, as a model for low-molecular-weight active agents, was determined and controlled by changing the cross-linking ratio. Han et al. (2000) also characterized the swelling ratio of cross-linked hydrogels that might affect the drug release rate. These included swelling ratio as affected by pH, salt and sugar concentrations, temperature of environments, and pK_a value of the free hydroxyl groups in the LPEPs.

The diffusion rate of proteins in hydrogels is an important property, both for characterizing solute–gel interactions and for designing novel bioactive applications of the hydrogel materials (Kong et al., 1997). Diffusion of macromolecules in polymeric matrixes may relate to various chemical and physical factors. Chemical factors may include hydrogen bonds, ionic bonds, electrostatic interactions, and hydrophobic interactions between macromolecule and matrix. Physical factors may include hydrodynamic radius, conformation of macromolecules, and the existence of binding sites. The physical factors may be more important to understanding the diffusion phenomenon of macromolecules in a nonionic polymeric matrix. Such factors include porosity, tortuosity, partition coefficient, steric hindrance, and frictional resistance (Kuu et al., 1992). The release rate of a solute (i.e., drug or agrochemical) related to these physical factors can be described by the experimental diffusivity (D_e). However, to discard the effect of solvent (water) viscosity, the diffusion rate is compared theoretically by the value of normalized diffusion coefficient (D_e/D_0). The D_0 is the diffusivity in water at infinite dilution, which can explain the diffusional transfer phenomenon of a moving substance. In nonionic gel

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systems with small solvent molecules (i.e., water), the hydrodynamic radius (R_h) and D_0 are related by the Stokes−Einstein equation:

$$D_0 = \frac{kT}{6\pi\mu R_h} \tag{1}$$

where k, T and μ are Boltzmann's constant, absolute temperature, and solvent viscosity, respectively. Kong et al. (1997) used eq 1 to determine the D_0 values of proteins and various sizes of surfactant micelles. The diffusion of nonionic micelles and sample proteins in the neutral hydrogels (agarose gel) followed eq 1. The D_e/D_0 values of proteins and nonionic micelles decreased with increasing agarose gel concentration. Because the diffusivity is proportional to inverse hydrodynamic radius (R_h) in dilute solution, with the assumption of the linear relationship between the molecular weight of globular protein and R_h^3, the diffusivity would depend linearly on the inverse cube root of molecular weight ($M_w^{-1/3}$). Therefore, in the dilute solution condition, such as compact globular proteins in loosely cross-linked hydrogels, the diffusion of sphere-shaped solute follows the inverse cube root of the molecular weight.

On the basis of the Stokes−Einstein theory, some modified equations were introduced to explain diffusion phenomena in the polymeric matrixes. D_0 in eq 1 can be interpreted that the major factors affecting diffusion are the ratio of temperature to solvent viscosity (T/μ) and the spatial hindrance (πR_h) of macromolecules by the cross-linked polymeric structure. Therefore, eq 1 could be generalized to eq 2, which is called the Rouse model (Cussler, 1997):

$$D_e = \frac{k_1 T}{N\zeta} = \frac{M_w^{\,n} k_2 T}{\zeta} \tag{?}$$

where k_1, k_2 are constants, N is the degree of polymerization which is proportional to the inverse molecular weight, n is the coefficient of molecular size effect, and ζ is the friction coefficient. In the case of condensed small spherical molecules in the loosely cross-linked matrixes, $n = -1/3$ in agreement with the assumption stated early. In the case of higher concentration of macromolecular solute $n = -1$, and the diffusion is proportional to inverse M_w (i.e., M_w^{-1}). Cussler (1997) also suggested the n value of $-1/2$ for the untangled random coil of solute. Therefore, the diffusivity of macromolecules in the polymeric structure would vary from -1 to $-1/3$ power of their molecular weight, depending on their structural characteristics and concentrations.

In swollen hydrogels or moderately cross-linked polymeric matrixes, the diffusion of a macromolecular solute is significantly affected by a friction effect compared to the diffusion in the dilute solution. Thus, the mesh size factor (size of the cavity) of the three-dimensionally cross-linked polymer gel matrixes should be considered in the diffusion model. Free volume fraction, which is the ratio of macromolecule size to the mesh size (or simply the ratio of the macromolecule size to the swelling ratio), could represent the mesh size factor of the polymer matrix as shown in eq 3 (Kuu et al., 1992; Hennink et al., 1997):

$$\frac{D_e}{D_0} = \Phi \exp\!\left(\frac{-k_3 R_h^{\,2}}{S-1}\right) \tag{3}$$

CLOSED SYSTEM

OPEN SYSTEM

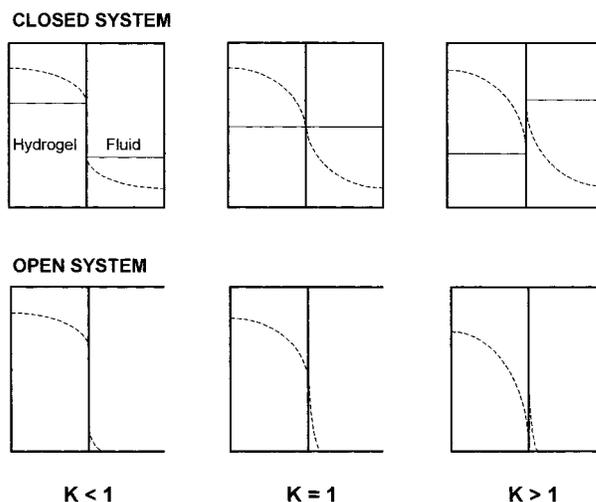$K < 1 \qquad\qquad K = 1 \qquad\qquad K > 1$

Figure 1. Concentration (y axis) distribution of diffusing substance in the hydrogel and the outside fluid (x axis). The dashed lines are for unsteady-state mass transfer during diffusion, and the solid lines of the closed systems show the concentration after attainment of equilibrium between hydrogel and fluid. $K = C_{\text{fluid}}/C_{\text{gel}}$.

where Φ, k_3 and S are the constant of mesh size factor, the constant of solute size factor, and swelling ratio of the matrix gel, respectively. Increasing swelling ratio increases diffusion rate. Because the negative inverse of swelling ratio ($-1/S$) has a first-order relationship to the diffusion rate (D_e/D_0), the smaller value of the swelling ratio affects the diffusion rate more significantly. Equation 3 shows that the logarithm of the diffusivity has a negative relationship to R_h^2, which can simply be assumed as $-M_w^{2/3}$ of diluted solute macromolecules.

The partition coefficient (K) provides the concentration relationship at the interface of two different media. It affects the concentration distribution of a moving substance during diffusion as well as after the equilibrium (Figure 1). Mass transfer flux at the interface between two media (i.e., hydrogel and fluid in this paper) is affected by the partition coefficient. The partition coefficient is the ratio of equilibrium concentrations in the two phases, which is caused by the chemical potential equilibrium. Therefore, unlike heat transfer, the solute can move from the lower concentration phase to the higher concentration phase at the interface to equilibrate the chemical potential, which is against kinetic or concentration equilibrium because the driving force of mass transfer is conventionally concentration difference. In the open system with an infinite volume of fluid, the higher partition coefficient (higher affinity to the fluid) accelerates diffusion because it increases the surface transfer flux at the interface of the gel and the fluid. Increased mass transfer flux at the interface caused by the higher partition coefficient reduces the concentration of solute at the hydrogel surface and increases the concentration gradient between the center and surface of the hydrogel. The larger gradient drives more diffusion of the solute in the hydrogel. However, it is difficult to measure the partition coefficient directly from the conventional mass-transfer system. It can be measured only after reaching the equilibrium of mass transfer with using the closed system (finite volume of the fluid).

The objectives of the research described in this paper were to (1) determine the diffusivity of proteins in the

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lactitol-based hydrogel and (2) determine the diffusion mechanism of the sample proteins in the hydrogel for verifying the factors influencing the release rate of protein delivery systems.

MATERIALS AND METHODS

Materials. α-Chymotrypsin (CTr), β-lactoglobulin (LG), bovine serum albumin (BSA), and γ-glubulin (gG) were purchased from Sigma Chemical Co. (St. Louis, MO). The molecular weights of these model proteins are 25K, 36K, 66K, and 150K, respectively. Chemicals for synthesizing hydrogels were purchased as described previously (Han et al., 2000).

Hydrogel Synthesis. Lactitol polyether polyol (LPEP) and chlorinated poly(ethylene glycol) bis(carboxymethyl) ether (PEGBCOCl) were synthesized by the method of Wilson et al. (1996). Lactitol-based polyether polyol cross-linked hydrogel was synthesized using the method described in previous work (Lin et al., 1998; Han et al., 1999). The cross-linking ratio (PEGBCOCl/LPEP) was 3:1. After reaction, the cross-linked hydrogel was washed with excess water and dried in a vacuum desiccator.

Production of Protein-Absorbed Hydrogels. Hydrogels were dried in a vacuum desiccator to remove water that might interfere with loading proteins. The dried hydrogels were placed overnight in 4 °C 0.01 N sodium phosphate buffer (pH 7.0) protein solution, which contained 0.1% (w/v) of the model proteins (CTr, LG, BSA, and gG). The hydrogels absorbed the protein solution and re-swelled. Before diffusion experiments, the hydrogel in protein solution was placed at room temperature for 0.5−1 h.

Protein-Release Model and Diffusivity Determination. Protein-loaded hydrogel is a homogeneous monolithic system that contains dissolved solute molecules in the gel matrix below the maximum solubility of the solute. For the hydrogel of half-thickness L, the fractional amount (M_t/M_∞) of released drug, which is the ratio of the amount released at time t (M_t) to the amount at time infinity (M_∞), is (Crank, 1975)

$$\frac{M_t}{M_\infty} = 1 - \sum_{n=0}^{\infty} \frac{8}{(2n+1)^2 \pi^2} \exp\left[-D(2n+1)^2 \pi^2 t/4L^2\right] \quad (4)$$

By including only the first term in the summation (Σ) series and performing a logarithmic transformation, Eq 4 can be simplified to eq 5, which applies only to the latter stages of diffusion (Crank, 1975):

$$\ln\left(1 - \frac{M_t}{M_\infty}\right) = \ln\frac{8}{\pi^2} - \frac{D_e \pi^2 t}{4L^2} \quad (5)$$

Using only experimental data for over 40% of the maximum release ($M_t/M_\infty > 0.4$), diffusivity, D_e, can be determined from the slope, $-D_e \pi^2/4L^2$, of the linear regression model of eq 5, which is eq 6:

$$Y_i = \beta_0 + \beta_1 x_i + \epsilon_i \quad (6)$$

where Y_i, x_i, β_0, β_1, and ϵ_i are $\ln(1 - M_t/M_\infty)$, time, intercept ($\ln 8/\pi^2$), slope ($-D_e \pi^2/4L^2$), and error, respectively. Because eq 5 is accurate only during the late period of release, theoretically eq 6 shows a good linearity between Y_i and x_i when fit to the experimental data for over 40% of the maximum release.

The protein-loaded hydrogels were cut to a square shape of 1.5-cm length and width. The thickness of the hydrogel was measured using a mechanical caliper which has an minimal measurement of 0.05 cm. The thickness of the hydrogels ranged from 0.35 to 0.4 cm, which is a minimum of 4 times lower than their length and width to satisfy the infinite slab condition according to the suggestion of Chorny and Krasuk (1966). The hydrogel was placed in 0.01 N sodium phosphate buffer (pH 7) solutions in a diffusion cell, maintained at 6, 25, 37, and 45 °C with a constant stirring speed (600 rpm). Figure
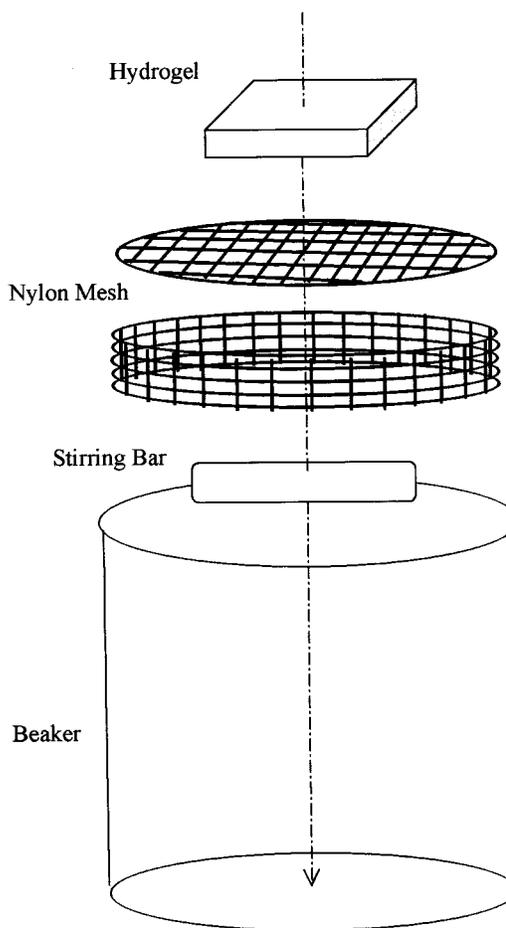

Figure 2. Migration cell.

2 shows the structure of the diffusion cell. Volume of the buffer solutions was 20 mL, which was more than 40 times the volume of the hydrogels. In the case of highly soluble solutes, Crank (1975) assumed that fluid volumes greater than 20 times that of the hydrogels is an infinite volume of fluid. The 40:1 volume ratio of fluid/hydrogel satisfied the assumption of Crank's infinite open system with near-zero concentration of boundary condition. The protein concentration was measured spectrophotometrically (Spectronic 1001, Bausch & Lomb) at 280 nm. The fluid was automatically collected by a peristaltic pump and circulated into the diffusion cell after the measurement of protein concentration through an in-line cuvette. The spectrophotometer displays three-decimal digits of absorbency measurement (minimum, 0.001) digitally and the maximal round-up error is in the forth decimal digit with 1 nm band-width.

The values of D_0 at 6, 25, 37, and 45 °C were calculated from the Stokes−Einstein equation (eq 1), using D_0 values at 20 °C in the literature (Smith, 1970) and assuming a constant value of $k/6\pi R_h$ for a given protein in eq 1, which is shown in eq 7.

$$D_0^{\,T} = \frac{k}{6\pi R_h^{\,T}}\frac{T}{\mu^T} = \frac{D_0^{\,20}\mu^{20}}{T^{20}}\frac{T}{\mu^T} = \frac{D_0^{\,20}\,T}{293\mu^T} \quad (7)$$

where the superscripts 20 and T indicate 20 °C and another temperature, respectively. Therefore, D_0 at any other temperature can be calculated from eq 7 with the value of $D_0^{\,20}$ and the viscosity of water (μ^T) at the temperature T. Calculated D_0 values for CTr, LG, BSA, and gG are listed in Table 1.

Swelling Ratio of Hydrogels. Washed hydrogels were placed in test tubes containing preheated water (6, 25, 37, 45, and 60 °C) in water baths, and the swelling ratio was measured with time to determine the shrinking rate at the

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Table 1. Infinite-Dilution Diffusion Coefficient (D_0) of Model Proteins at Various Temperatures[a]

protein	D_0^6	D_0^{25}	D_0^{37}	D_0^{45}	D_0^{20b}
α-chymotrypsin (CTr)	6.60×10^{-7}	1.17×10^{-6}	1.56×10^{-6}	1.86×10^{-6}	1.02×10^{-6}
β-lactoglobulin (LG)	4.84×10^{-7}	8.54×10^{-7}	1.14×10^{-6}	1.36×10^{-6}	7.48×10^{-7}
bovine serum albumin (BSA)	3.98×10^{-7}	7.02×10^{-7}	9.41×10^{-7}	1.12×10^{-6}	6.15×10^{-7}
γ-globulin (gG)	2.65×10^{-7}	4.68×10^{-7}	6.27×10^{-7}	7.47×10^{-7}	4.10×10^{-7}

[a] Obtained by eq 7. [b] From Smith, 1970.

temperature. The hydrogels were gently wiped with tissue paper to remove excess water on the surface, then weighed (W_{wet}). After drying in a vacuum desiccator for at least 48 h, the dried hydrogels were weighed again (W_{dry}). The swelling ratio (S) was calculated by the following equation.

$$S = \frac{W_{wet} - W_{dry}}{W_{dry}} \tag{8}$$

The effects of heating time and temperature on the swelling ratio were examined by plotting the swelling ratio (S) versus heating time (t) and temperature (T). The rates of swelling ratio reduction (shrinking ratio) were obtained from the slope of the S vs t plot.

Viscosity of Protein and Buffer Solutions. Viscosities of distilled water, 0.01 N sodium phosphate buffer (pH 7.0) solution, 0.5% LG solution, and 0.5% BSA solutions were measured by a capillary viscometer at 25 , 37 , 45, and 60 °C. Protein solutions (0.5% LG and BSA) were formulated with 0.01 N sodium phosphate buffer (pH 7.0). The passing times of the sample solutions through the capillary viscometer were measured. Apparent viscosity (μ) was obtained from the ratio of the measured passing time of sample solutions to that of pure water. The absolute viscosity of pure water at different temperatures was obtained from the literature (Weast, 1979).

Partition Coefficients of Proteins between Hydrogel and Buffer Solution. Partition coefficients of LG and BSA were measured at 6, 25, 37, 45, and 60 °C to determine the temperature effect. The partition coefficient, K, of proteins was defined as shown in eq 9.

$$K = \frac{C_{soln}}{C_{gel}} \tag{9}$$

where C_{soln} and C_{gel} are the concentration of the protein in the solution and in the hydrogel, respectively. The concentration of the protein in the hydrogel was defined as shown in eq 10.

$$C_{gel} = \frac{M_{gel}}{V_{gel}} = \frac{M_0 - M_{soln}}{V_{gel}} = \frac{(C_0 - C_{soln}) V_{soln}}{V_{gel}} \tag{10}$$

where M_{gel}, M_{soln}, and M_0 are the mass of the protein in the hydrogel and in the solution, and the total mass of the protein, respectively. V_{gel}, V_{soln}, and C_0 are the volume of the hydrogel, the volume of the solution, and initial concentration of the proteins in the solution, respectively. Therefore, the partition coefficient of the protein can be calculated by eq 11 after measuring the volume of the hydrogel (V_{gel}) and solution (V_{soln}), and the absorbance of the protein solution without hydrogels (A_0) and of the solution (A_{soln}) with hydrogels at 280 nm.

$$K = \frac{C_{soln}}{C_{gel}} = \frac{C_{soln}}{(C_0 - C_{soln}) V_{soln}/V_{gel}} = \frac{A_{soln} V_{gel}}{(A_0 - A_{soln}) V_{soln}} \tag{11}$$

Proteins (LG and BSA) were dissolved into known volumes of sodium phosphate buffer solutions (V_{soln}) with concentrations of 0.02% (w/v). The absorbances (A_0) of the protein solutions were measured at 280 nm spectrophotometrically. Hydrogels were placed in the protein solutions and stored at 6, 25, 37, 45, and 60 °C for 24 h to reach the equilibrium. After measuring the total volume (V_{total}) of the solution with hydrogels, the volumes of the hydrogels were calculated from the difference of V_{total} and V_{soln}. The protein solutions were sampled

and equilibrated at room temperature for 1 h. The absorbances of the sampled solutions (A_{soln}) were measured by spectrophotometer at 280 nm, and then the partition coefficients of LG and BSA were obtained by eq 11.

RESULTS AND DISCUSSION

LPEP Hydrogels. Hydrated hydrogels with 3:1 molar ratio of PEGBCOCl/LPEP were strong enough to be handled, and were transparent even after absorbing protein solutions. Dried hydrogels were very strong and showed a rubber-like consistency. Protein-loaded hydrogels were slightly hazy after drying. The dried hydrogel shrank to less than half of the hydrated hydrogel volume. However, protein-loaded hydrogel did not shrink as much as the empty hydrogel after drying. This result implies that the protein molecules may interfere with the polymer−polymer interaction during and/or after dehydration. Water molecules are bound to the hydrated hydrogel polymeric structure because of the hydrophilic nature of the hydrogel. Thus, bound water molecules interfere with the hydrophilic polymer−polymer interaction of the hydrated hydrogel. During drying the water molecules are removed from the hydrogel. Dehydration removes the water and thus allows hydrophilic polymer and polymer interaction in the hydrogel. However, in the case of dry protein-loaded hydrogels, protein molecules may interfere with the polymer−polymer interaction due to hydrophilic interactions with polymers. The result is a greater distance between polymers, resulting in the larger hydrogel volume compared to that of the dry empty hydrogel.

Diffusion of Proteins. After complete release of proteins, the maximum absorbency of the fluid ranged from 0.1 to 0.3. BSA, LG, CTr, and gG had maximal absorbency values of 0.1, 0.1, 0.2, and 0.3, respectively, in the fluid. Increasing the molecular weight (M_w) of proteins slowed diffusion at 37 °C (Figure 3A). The larger protein molecules (e.g., gG) moved more slowly in the hydrogel and were released more slowly into the buffer solution. The smallest protein (i.e., CTr) showed the fastest diffusion. The mass transfer of the larger protein molecules is likely more hindered by the polymeric network during diffusion through the hydrogel to the buffer solution.

Figure 3B shows the linear relationship found between the logarithmic-transformed fractional release of the proteins for over 40% of maximum release and the square root of time, consistent with eq 5. The protein diffusivities (D_e) obtained by applying eq 5 to Figure 3B are 8.65×10^{-6} ($\pm 5.95 \times 10^{-8}$), 4.48×10^{-6} ($\pm 9.38 \times 10^{-7}$), 2.86×10^{-6} ($\pm 3.55 \times 10^{-7}$), and 1.29×10^{-6} ($\pm 1.02 \times 10^{-6}$) cm²/sec for CTr, LG, BSA, and gG, respectively, at 37 °C.

Figure 4 shows the relationships between the diffusivity of proteins and their molecular weights at 37 °C. Experimental diffusivity (D_e) and normalized diffusivity (D_e/D_0) decreased with increasing molecular weight (Figures 4A and D). Both D_e and D_e/D_0 showed a significant linear relationship to the inverse of molec-
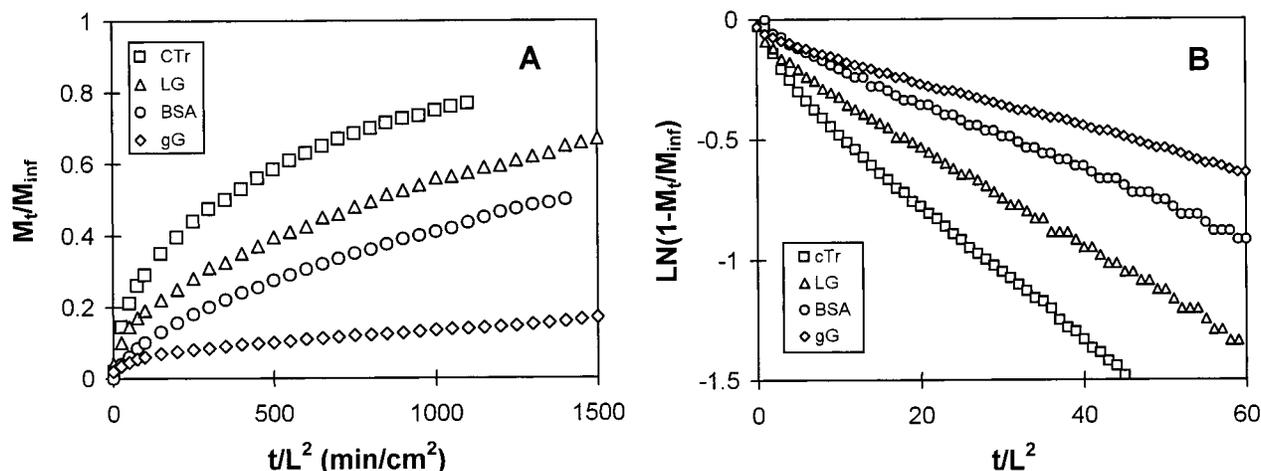
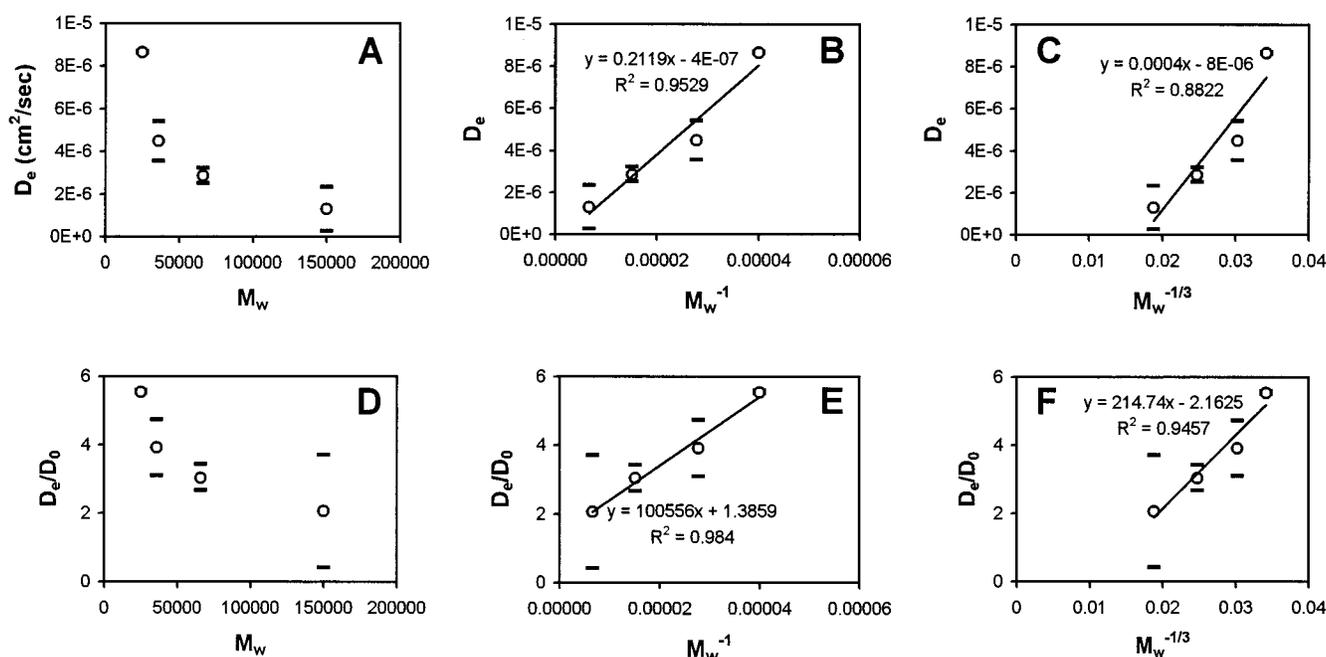**Figure 3.** Release profiles of proteins from LPEP hydrogels at 37 °C.

Figure 4. Effects of molecular weights of proteins on the experimental diffusivity (D_e) and the normalized diffusivity (D_e/D_0) at 37 °C. Bars indicate the standard deviations of 4 or 5 replications.

ular weight (M_w^{-1}) (Figures 4B and E) and inverse cube root of molecular weight ($M_w^{-1/3}$) (Figures 4C and F). Generally, D_e/D_0 showed better linearity to M_w^{-1} and $M_w^{-1/3}$ than those of the experimental diffusivity (D_e). In particular, D_e/D_0 had much better linearity to $M_w^{-1/3}$ ($R^2 = 0.9457$) than D_e to $M_w^{-1/3}$ ($R^2 = 0.8822$) (Figures 4F and C). The higher value of R^2 for D_e/D_0 vs $M_w^{-1/3}$, compared to R^2 of D_e vs $M_w^{-1/3}$, may imply that D_e/D_0 is more appropriate to describe the diffusion phenomena than D_e alone. The cube root of molecular weight relates to the three-dimensional character of proteins. Therefore, this result confirms the suggestion in the Introduction that the diffusion of compact globular molecules follows a linear relationship to the inverse cube root of molecular weight (eq 2).

Infinite-dilution diffusivity (D_0) represents diffusional molecular movement that is caused by concentration difference of the molecule between two positions in water. Physical mixing and other chemical interaction and repulsion are not involved in determining D_0, other than the effects of stationary water on the diffusion of the moving substance. However, the LPEP hydrogel and

buffer system was vigorously stirred to remove surface resistance (boundary film layer of the mass transfer) of the LPEP hydrogels during diffusion to obtain D_e of proteins. This forced convection of the outside fluid may have enhanced mass transfer of protein inside the hydrogel, resulting in higher values of D_e compared to D_0, which was measured without stirring. Without forced convection, theoretically, D_e of protein in the cross-linked hydrogel cannot exceed the value of D_0, because of the spatial hindrance and molecular interactions by the hydrogel polymers. However, D_e/D_0 values were ~2 and above in this paper, perhaps because of the enhancing effect of the forced convection of the outside fluid on the protein diffusion in the hydrogel. The LPEP hydrogels have a swelling ratio of 20 to 25. Thus, they contain water 20 to 25 times their weight. Therefore, the hydrogels contain 3.9−4.7% of LPEP and 95.3−96.1% of water. Vigorous fluid stirring generated the high forced convection of the outside fluid and also may have circulated the water molecules positioned in the hydrogels. These forced convection conditions differ from the circulatory conditions in animal bodies. There-
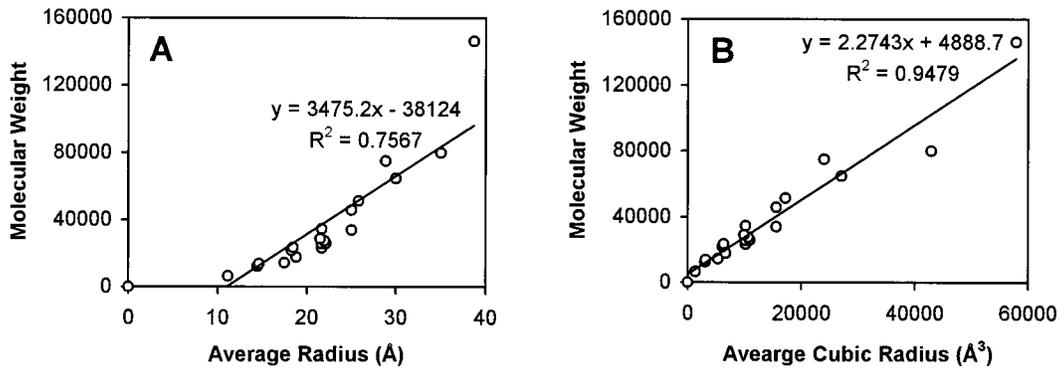
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Figure 5. Relationship between molecular weight and radius of the dehydrated crystal protein structure.
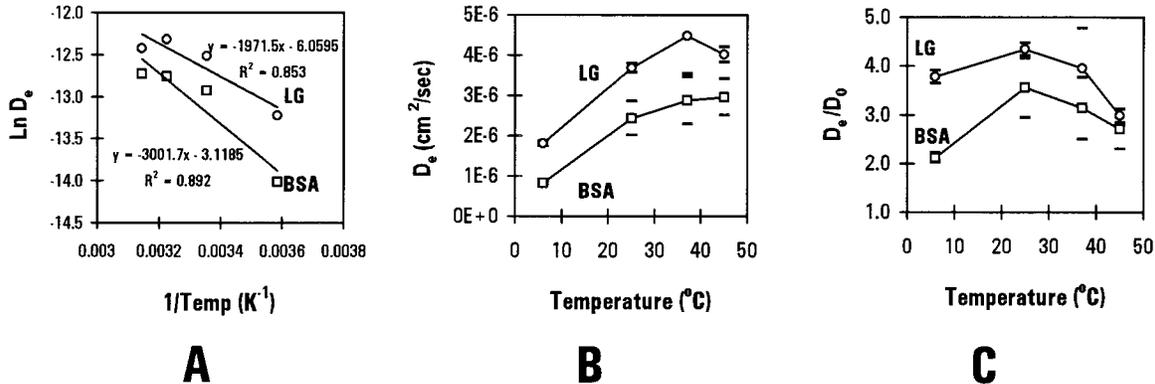

Figure 6. Effect of temperature on the diffusion of β-lactoglubulin (LG) and bovine serum albumin (BSA). Bars indicate the standard deviations of 4 or 5 replications.

fore, the diffusion results in the in vivo situation may vary from our results.

Extrapolation of the linear line of Figure 4F to the X-axis intercepts a $M_w^{-1/3}$ value of 0.01 at a D_e/D_0 value of zero, which means no diffusional mass transfer occurs. Therefore, a M_w of 1000K (which is equivalent to $M_w^{-1/3}$ of 0.01) is the critical molecular weight above which no macromolecule movement occurs in the LPEP hydrogel. Because D_e/D_0 accounts for eliminating the effect of water viscosity so that the X-axis of Figure 4F is the molecular weight of dehydrated protein molecules, 1000K indicates the exact critical size of protein molecule movement without hydrating water. Thus, the cavity size of the LPEP hydrogel may be the same size as the dehydrated 1000K M_w globular proteins. However, extrapolation of the line of Figure 4C to the X-axis intercepts $M_w^{-1/3}$ value of 0.017, which is equivalent to a M_w value of 200K. The experimental diffusivity (D_e) includes the effect of hydrating water molecules and viscosity, so that the 200K M_w is the hydrodynamic molecular size. Proteins with less than 200K equivalent M_w can move through the LPEP hydrogel in their hydrated conformation. Therefore, the cavity size of the LPEP hydrogel is the same size as a hydrated protein molecule of 200K M_w with surrounded hydrating water layer. Because the LPEP hydrogels for the protein release were the same materials, the above two estimates of the cavity sizes have to be the same value. Therefore, it is suggested that the size of dehydrated protein molecules of 1000K M_w is equivalent to that of the hydrated protein molecules of 200K M_w with surrounding bound water layer. The difference between 1000K and 200K may be caused by water viscosity and the water-shield (hydrodynamic) layer around the protein molecule, which moves together with the protein

molecule. Molecular weights of globular proteins increase 5 times after hydration and their radii may increase 1.25 times ($5^{-1/3}$) after hydration. The hydrodynamic radius of protein would be 1.25 times the radius of dehydrated protein molecule at 37 °C.

To estimate the average cavity size of the LPEP hydrogel, M_w vs average radii of various protein molecules was plotted. Figures 5A and B were generated from Squire and Himmel's data (1974) of the molecular weight and the radius. Figure 5B shows a good fit ($R^2 = 0.9479$) between M_w and cube radius (R^3) of protein molecules of dehydrated crystal structure compared to Figure 5A ($R^2 = 0.7567$) which uses the linear scale of the radius. From the linear regression result ($M_w = 2.2743\,R + 4888.7$), the radius of the 1000K M_w protein was calculated to be 75.9 Å. Therefore, the cavity width of the LPEP hydrogel may be 15.1 nm (75.9 Å × 2), which is the diameter of the 1000K M_w protein.

Higher temperature resulted in faster protein diffusion. However, diffusion did not follow the Arrhenius equation above 37 °C. Figure 6A does not show a good linearity between ln D_e and inverse temperature, especially at the temperature range above 37 °C (i.e., below T^{-1} of 0.0032). This stability of experimental diffusion rate (D_e) around body temperature may provide a benefit to clinical use of the protein delivery system. The normalized diffusion rate (D_e/D_0) decreased with increasing temperature above 25 °C (Figure 6C). Protein molecules were more hindered in the LPEP hydrogels above 25 °C with increasing temperature.

Swelling Ratio with Temperature. At and above 37 °C, swelling ratio decreased with increasing temperature and heating time. This suggests that greater thermal energy liberates more water molecules which were hydrated to the polymeric hydrogel structure
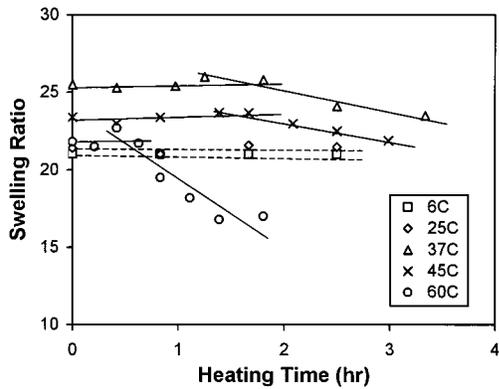
Figure 7. Swelling ratio changes with changing heating time and temperature.

(Figure 7). The hydrogel shrunk faster at higher temperature. At 60 °C, the swelling ratio decreased fastest with the steepest slope, whereas at 6 °C and 25 °C, the hydrogel did not show a swelling ratio change. At 37 and 45 °C, the swelling ratio started to decrease after 2 h of heating. These swelling ratio changes may affect the diffusion rate of proteins. In the cases of LG and BSA release experiments, it took about 2−3 h for complete depletion of proteins from the hydrogels, which is longer than the period starting decrease of the swelling ratio at 37 and 45 °C. Because eqs 5 and 6 used protein concentration data after 40% of migration until depletion (100% migration) to calculate the diffusivity

(D_e), the obtained diffusivity may be affected by the hydrogel shrinking after 2 h at 37 and 45 °C. This hydrogel shrinking may reduce the free cavity size of the hydrogels and may decrease the diffusivity (D_e/D_o) above 37 °C (Figure 6C).

Viscosity of Protein Solutions. There was no viscosity difference found between pure water and sodium phosphate buffer solution. From 6 to 60 °C, they showed identical viscosities (Figure 8). Thus, types of solution (water and buffer) did not affect the diffusion rate, because the fluid viscosity was not altered by changing the solvent from water to buffer.

Heating time of protein solution did not affect the viscosity, although increasing temperature decreased the viscosities of protein solutions. Figure 9A shows little change in viscosity with increasing heating time at a certain temperature. The 0.5% protein solutions of LG and BSA did not show any difference in their viscosities. Even temperature (60 °C) above the denaturation temperature of BSA did not affect the viscosity change until 2 h. The viscosity of 0.5% LG and BSA in sodium phosphate buffer was identical to that in water. Figure 9B also shows that viscosity of the protein solutions and water were the same with respect to the temperature. This result suggests that the hydrodynamic radii (R_h) of model proteins are not changed significantly by heating within the experimental temperature range (6 − 60 °C). This conclusion agrees with the assumption of eq 7 that $k/6\pi R_h$ would be a constant at any temperature.
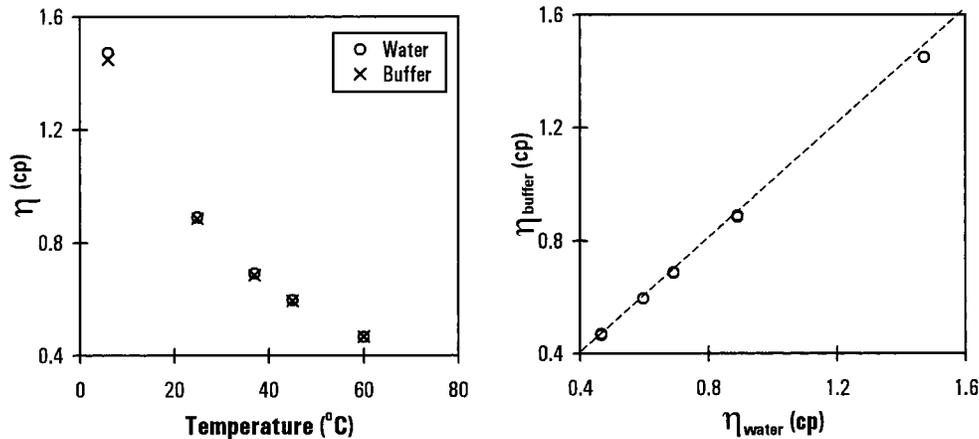

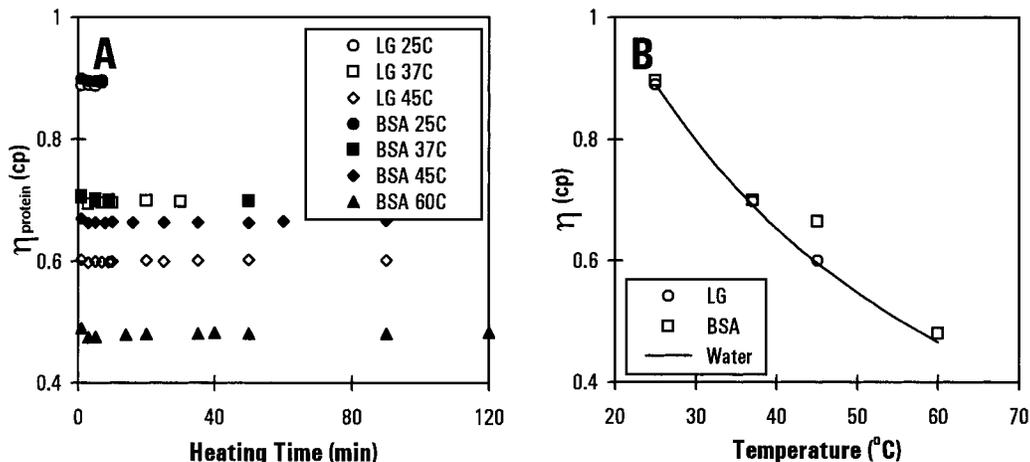**Figure 8.** Viscosities of pure water and sodium phosphate buffer solution.

Figure 9. Viscosities of protein solutions (LG and BSA) with respect to heating time and temperature.

Protein Release from Hydrogel

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Table 2. Partition Coefficients ($K = C_{Fluid}/C_{Gel}$) of β-Lactoglobulin (LG) and Bovine Serum Albumin (BSA)

temperature (°C)	partition coefficient $(K)^a$	
	LG	BSA
6	>10	>10
25	∼10	>10
37	∼10	>10
45	∼10	∼10
60	2.3	2.1

^{a} $K = 10$ means that 90.9% of protein is dissolved in the fluid and 9.1% is in the hydrogel. Because 9.1% is insignificant compared to the experimental error, the partition coefficients larger than 10 and near 10 are reported as >10 and ∼10, respectively.

Partition Coefficients of Proteins. The test proteins (LG and BSA) had high affinity to the surrounding water (Table 2). Because of the high partition coefficient of the proteins to the water, there exists a discrete concentration change at the hydrogel–water interface consistent with the $K > 1$ profile shown in Figure 1 for an open system. Because of the high partition coefficient, Crank's model and the diffusivity determination in this paper were not affected by the distribution of proteins at the interface. Because of the high partition coefficient, the experimental conditions of the mass transfer were consistent with the boundary conditions assumed in the model (eq 4 and 5), which were zero concentration at the hydrogel interface.

CONCLUSIONS

Incorporated proteins are released from the cross-linked hydrogel into the surrounding fluid at specific release rates. The mass transfer follows the theory of hindered diffusion. The diffusion rates (D_e/D_0) of proteins increase with increasing temperature from 4 to 25 °C, but they decrease with increasing temperature above 25 °C, perhaps because of the hydrogel shrinking at the high temperature. At 37 °C, the experimental release rates of proteins (D_e) were not significantly different from those at 25 °C and 45 °C. This stability of release rate at the body temperature is a potential advantage characteristic of the cross-linked hydrogel for the clinical use of protein drug delivery systems.

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