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Swelling and Dissolution of β-Lactoglobulin Gels in Alkali

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It is well documented in the literature that during the dissolution of whey protein gels in alkali, the gels swell to a great extent. However, the relevance of the swelling step in the dissolution process of the protein gel remains unknown. In the present article we present a systematic study on the swelling of β-lactoglobulin gels at different alkaline pH and ionic strengths. The equilibrium swelling degree at different conditions has been modeled using a simple model developed for polyelectrolyte gels, modified to take into account the ionization of the residues in a protein. The model can describe the swelling behavior of the gels over a wide range of conditions, but it underpredicts the equilibrium swelling under conditions close to those when dissolution is observed. Dissolution is only noticeable above pH 11.5−12 and only for those gels that are swollen over a minimum degree, suggesting the existence of a dissolution threshold.

Introduction

The heat-induced gelation of whey proteins has been extensively reported in the literature,[1,2] and β-lactoglobulin (βLg) in particular has been widely used as a model protein.[3−5] One area involving whey gels that has not received much detailed attention is in the cleaning of dairy plants. Pasteurization and sterilization are primarily achieved by heat treatment, which results in the formation of fouling deposits on heat transfer equipment and other surfaces.[6] The deposits consist of a mixture of proteins, fats, sugars, and mineral salts.[7] However, whey proteins, and particularly βLg, constitute the bulk of fouling deposits (>50 wt %) in lower temperature operations,[8] and therefore the deposits can be considered to be primarily heat-induced gels.[9] The removal of such deposits is critical to the dairy industry in terms of downtime, energy, cleaning, hygiene, and waste treatment costs. Cleaning is usually performed by circulating alkali solutions through the equipment in cleaning-in-place (CIP) operations. Several models have been proposed for the cleaning of proteinacious deposits,[10] but the mechanism is still not well understood due to the complexity of the problem.

The dissolution of whey gels involves several steps. First, there is the diffusion of the alkali from the bulk solution into the gel. This step is known to be fast,[11] and therefore the penetration depth of the alkali in the gel can be observed.[12] The increased interprotein repulsion in the NaOH penetration zone causes the deposit to swell significantly.[13,14] Thereafter, the deposit starts to dissolve. The parameters that determine the onset of dissolution during the swelling stage are not known. The next steps in the dissolution process are the cleavage reactions of the interprotein cross-links,[15] the disentanglement of the protein clusters from the penetration zone,[16] and the diffusion of the proteins from the boundary layer adjacent to the deposit into the bulk solution.[17]

Despite the fact that swelling is always observed during dissolution (to a higher or lower degree), a detailed study of the swelling of whey gels has not been performed, and therefore, the implications of the swelling step in dissolution are not clear. In the present study we have analyzed the effect of pH and ionic strength on the equilibrium swelling degree (Q) of βLg gels, which have also been used as model gels in dissolution experiments.[15,16] Swelling studies of protein-based hydrogels are scarce in the literature,[18−21] although there is recent interest on the swelling of whey gels for controlled release applications.[22,23] In the present paper, we present a simple model for the swelling of protein gels incorporating the effect of solvent pH and ionic strength and consider its implications for advancement in the understanding of the gel dissolution process.

Theory

Model Derivation. The model is adapted from that proposed by Brannon-Peppas and Peppas (BPP)[24,25] for the swelling of polyelectrolyte hydrogels containing one single type of anionic group in the polymer chain. For brevity, we begin the model derivation from the statement of swelling equilibrium that relates the difference between the ionic contributions from the chemical potential inside the gel, $(\Delta\mu_1)_{\text{ion}}$, and in solution, $(\Delta\mu_1{}^*)_{\text{ion}}$, to the sum of the contributions to the chemical potential of the mixing, $(\Delta\mu_1)_{\text{mix}}$, and elastic forces, $(\Delta\mu_1)_{\text{el}}$, due to polymer−swelling agent interactions[24]

$$(\Delta\mu_1{}^*)_{\text{ion}} - (\Delta\mu_1)_{\text{ion}} = (\Delta\mu_1)_{\text{mix}} + (\Delta\mu_1)_{\text{el}} \tag{1}$$

The common notation is used, where subscripts 1 refer to the solvent and 2 to the polymer or protein, respectively, while the superscript asterisk refers to the region outside the gel, i.e., the solution, and the absence of the superscript denotes the region inside the hydrogel. The present model focuses on the derivation of the ionic contributions for amphiphilic polymers, such as proteins, with several types of positively and negatively charged groups. The ionic contribution to the chemical potential is related to the difference in the ion concentrations in the hydrogel and in solution, where it is assumed that activities can be approximated by concentrations[26]

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$$(\Delta\mu_1^*)_{\text{ion}} - (\Delta\mu_1)_{\text{ion}} = \upsilon_{\text{m},1}RT\sum_{j}^{\text{all-ions}}(c_j - c_j^*) = \upsilon_{\text{m},1}RT(c_+ + c_- - c_+^* - c_-^*) \tag{2}$$

where $\upsilon_{\text{m},1}$ is the molar volume of the solvent, R is the gas constant, T is the absolute temperature, c_j is the molar concentration of ion j, and c_+ and c_- are the total molar concentrations of cations and anions, respectively. Considering that only 1:1 electrolytes are present in the system, the cationic and anionic concentrations in solution are due only to mobile ions, $c_+^* = c_-^* = c_\text{s}^*$, where c_s^* is the molar electrolyte concentration in solution. However, the cationic and anionic concentrations inside the hydrogel are due to both the presence of the mobile electrolyte and the fixed charges in the protein

$$c_+ = c_\text{s} + \sum_{p}^{\text{positive}} i_p c_p \quad p = \text{His, Lys, and Arg} \tag{3}$$

$$c_- = c_\text{s} + \sum_{n}^{\text{negative}} i_n c_n \quad n = \text{Asp, Glu, Cys, Tyr, Ser, and Thr} \tag{4}$$

where c_s is the molar electrolyte concentration in the hydrogel, c_p and c_n are the total molar concentrations of an ionizable residue p or n in the hydrogel, and i is the degree of ionization of the residue. Subscript p refers to amino acids with positively charged side chains, and n to negatively charged amino acids. The total molar concentration of an ionizable residue k in the hydrogel is defined as

$$c_k = \frac{\phi_{2,\text{s}}N_k}{\upsilon_{\text{sp},2}M_\text{w}} \quad k \,\forall\, n,p \tag{5}$$

where $\phi_{2,\text{s}}$ is the polymer volume fraction in the swollen gel, N_k is the number of residues k present in one protein molecule, $\upsilon_{\text{sp},2}$ is the specific volume of the protein, and M_w is the molecular weight of the protein. The degree of ionization of a residue is related to the pH and to the basicity constant, $K_{\text{b},k}$, through eq 6 for positively charged amino acids and eq 7 for negatively charged ones

$$i_p = \frac{1}{10^{\text{pH}-14}K_{\text{b},p} + 1} \tag{6}$$

$$i_n = \frac{K_{\text{b},n}}{10^{14-\text{pH}} + K_{\text{b},n}} \tag{7}$$

Assuming that there is perfect cancellation between positive and negative fixed charges in the protein, combining eqs 2−5 we obtain

$$(\Delta\mu_1^*)_{\text{ion}} - (\Delta\mu_1)_{\text{ion}} = \upsilon_{\text{m},1}RT\left[\frac{\phi_{2,\text{s}}}{\upsilon_{\text{sp},2}M_\text{w}}\left(\sum_{p}^{\text{positive}} i_pN_p - \sum_{n}^{\text{negative}} i_nN_n\right) - 2(c_\text{s}^* - c_\text{s})\right] \tag{8}$$

We then define the net charge in the protein, $N_\pm$, and the total charge in the protein, N_T, as

$$N_\pm = \left|\sum_{p}^{\text{positive}} i_pN_p - \sum_{n}^{\text{negative}} i_nN_n\right| \tag{9}$$

$$N_\text{T} = \sum_{p}^{\text{positive}} i_pN_p + \sum_{n}^{\text{negative}} i_nN_n \tag{10}$$

The net concentration of fixed charges inside the hydrogel is

$$c_\pm = \frac{\phi_{2,\text{s}}N_\pm}{\upsilon_{\text{sp},2}M_\text{w}} = a_0\phi_{2,\text{s}} \quad \text{where} \quad a_0 \equiv \frac{N_\pm}{\upsilon_{\text{sp},2}M_\text{w}} \tag{11}$$

Thus eq 8 can be written as

$$(\Delta\mu_1^*)_{\text{ion}} - (\Delta\mu_1)_{\text{ion}} = \upsilon_{\text{m},1}RT[c_\pm - 2(c_\text{s}^* - c_\text{s})] \tag{12}$$

Following refs 27 and 28, we can write eq 12 as

$$(\Delta\mu_1^*)_{\text{ion}} - (\Delta\mu_1)_{\text{ion}} = \upsilon_{\text{m},1}RT\left(\frac{{c_\pm}^2}{c_\pm + 4I}\right) \tag{13}$$

where I is the ionic strength of the solution. The low-salt limit ($c_\pm \gg 4I$) and high-salt limit ($c_\pm \ll 4I$) regimes yield the following relations[24,27]

$$(\Delta\mu_1^*)_{\text{ion}} - (\Delta\mu_1)_{\text{ion}} = \upsilon_{\text{m},1}RTc_\pm \quad c_\pm \gg 4I \tag{14}$$

$$(\Delta\mu_1^*)_{\text{ion}} - (\Delta\mu_1)_{\text{ion}} = \upsilon_{\text{m},1}RT\frac{{c_\pm}^2}{4I} \quad c_\pm \ll 4I \tag{15}$$

The BPP model considers both regimes separately,[25] while some other studies employ only the low-salt[29−31] or the high-salt regime.[32−35] Here, the full form of eq 13 will be used to describe the ionic contribution to the chemical potential difference. We can rewrite eq 13 using eq 11, yielding the final expression for the ionic contribution to the chemical potentials

$$(\Delta\mu_1^*)_{\text{ion}} - (\Delta\mu_1)_{\text{ion}} = \upsilon_{\text{m},1}RT\left(\frac{{a_0}^2{\phi_{2,\text{s}}}^2}{a_0\phi_{2,\text{s}} + 4I}\right) \tag{16}$$

The term $(\Delta\mu_1)_{\text{mix}}$ from eq 1 is described by the Flory−Huggins lattice theory,[36] while the affine network model[36] is used to describe the elastic component, $(\Delta\mu_1)_{\text{el}}$, as explained in detail elsewhere.[24] Non-Gaussian effects in $(\Delta\mu_1)_{\text{el}}$ due to highly stretched chains are not considered because the experimental degrees of swelling are not large ($Q = {\phi_{2,\text{s}}}^{-1} < 30$).[37] Therefore, the final equation at swelling equilibrium is

$$\upsilon_{\text{m},1}\left(\frac{{a_0}^2{\phi_{2,\text{s}}}^2}{a_0\phi_{2,\text{s}} + 4I}\right) = \ln(1 - \phi_{2,\text{s}}) + \phi_{2,\text{s}} + \chi{\phi_{2,\text{s}}}^2 + \nu_\text{e}\upsilon_{\text{m},1}\phi_{2,\text{r}}\left[\left(\frac{\phi_{2,\text{s}}}{\phi_{2,\text{r}}}\right)^{1/3} - \frac{1}{2}\left(\frac{\phi_{2,\text{s}}}{\phi_{2,\text{r}}}\right)\right] \tag{17}$$

where χ is the Flory−Huggins polymer−solvent interaction parameter, $\phi_{2,\text{r}}$ is the polymer volume fraction in the relaxed gel (before swelling), and ν_e is the cross-linking density. χ usually depends on the polymer concentration, and a first-order Taylor expansion is used here

$$\chi(\phi_{2,\text{s}}) = \chi_0 + \chi_1\phi_{2,\text{s}} + \cdots \tag{18}$$

Determination of Model Parameters. An approach similar to one the used by Güven and co-workers[33,34,38] is applied to determine the Flory−Huggins interaction parameter and ν_e in the gel. We define A and B as follows

$$A \equiv \upsilon_{\mathrm{m},1}\left(\frac{{a_0}^2}{a_0\phi_{2,\mathrm{s}} + 4I}\right) - {\phi_{2,\mathrm{s}}}^{-2}\ln(1 - \phi_{2,\mathrm{s}}) - {\phi_{2,\mathrm{s}}}^{-1} \tag{19}$$

$$B \equiv {\phi_{2,\mathrm{s}}}^{-2}\left[\left(\frac{\phi_{2,\mathrm{s}}}{\phi_{2,\mathrm{r}}}\right)^{1/3} - \frac{1}{2}\left(\frac{\phi_{2,\mathrm{s}}}{\phi_{2,\mathrm{r}}}\right)\right] \tag{20}$$

Considering a first-order expansion of χ (eq 18), eq 17 can be linearized,[33,34,38] and the parameters χ_0, χ_1, and ν_e are found with an appropriate optimization method

$$A - \nu_e\upsilon_{\mathrm{m},1}\phi_{2,\mathrm{r}}B = \chi(\phi_{2,\mathrm{s}}) = \chi_0 + \chi_1\phi_{2,\mathrm{s}} \tag{21}$$

$$A - \chi_1\phi_{2,\mathrm{s}} = \chi_0 + \nu_e\upsilon_{\mathrm{m},1}\phi_{2,\mathrm{r}}B \tag{22}$$

Hydrogen Ion Equilibrium. The hydrogen ion equilibrium curve depends on the ionic concentration,[39] the denaturing conditions,[40] and the different βLg genetic variants.[41] In the present study, the hydrogen ion equilibrium of βLg used was determined for better accuracy; the protein was used as received, i.e., it was not purified further. A similar procedure to the classical method[39] has been used here. The final hydroxide concentration c^*_{OH} in a βLg solution after concentrated NaOH is added is calculated with the following equation

$$c^*_{\mathrm{OH,ad}} = c^*_{\mathrm{H},0} - c^*_{\mathrm{H}} + c^*_{\mathrm{OH}} - c^*_{\mathrm{OH},0} + \sum_k (c^*_{k,0} - c^*_k) \tag{23}$$

where $c^*_{\mathrm{OH,ad}}$ is defined as the moles of NaOH added divided by the final volume of the solution, c^*_{H} is the proton concentration, and c^*_k are the molar concentrations of the different ionizable groups in their acid form. The subscript 0 refers to the concentration before the addition of the caustic. In the present work, only the neutral to alkaline range will be studied. Therefore, the subscript k is only for the His, Cys, Tyr, Lys, Arg, and Ser−Thr side chains involved in acid−base reactions at these conditions. c^*_k is calculated with the total protein concentration, $c^*_{\beta\mathrm{Lg}}$, the number of residues k present in one βLg molecule, N_k, and the degree of ionization of the k residue, where $K_{\mathrm{b},k}$ is the basicity constant of the kth equilibrium

$$c^*_k = c^*_{\beta\mathrm{Lg}}N_k\frac{1}{1 + K_{\mathrm{b},k}c^*_{\mathrm{OH}}} \tag{24}$$

Equation 23 can be simplified as $c^*_{\mathrm{OH,ad}}$ is always greater than 10^{-3} M, so $c^*_{\mathrm{OH,ad}} \gg c^*_{\mathrm{H},0} - c^*_{\mathrm{H}} - c^*_{\mathrm{OH},0}$. Rearranging eq 23 with eq 24 yields the following relationship to calculate the mean number of protons dissociated per protein molecule, as follows

$$\frac{c^*_{\mathrm{OH,ad}} - c^*_{\mathrm{OH}}}{c^*_{\beta\mathrm{Lg}}} \cong \sum_k N_k\left(\frac{1}{1 + K_{\mathrm{b},k}c^*_{\mathrm{OH},0}} - \frac{1}{1 + K_{\mathrm{b},k}c^*_{\mathrm{OH}}}\right) \tag{25}$$

Experimental Methods

Materials. β-Lactoglobulin (βLg) was kindly donated by Davisco Foods International, Inc. (Lesueur, MN). The composition of the powder as given by the manufacturer (lot no. JE 003-3-922) was 5.8% moisture; the solids (d.b.) were 97.4% protein of which 95.0% was βLg, 0.1% fats, and 2.4% ash. βLg powder and solutions were stored at 4 °C in airtight containers. Reagents used were of analytical grade (Sigma, Fisher) and were used as received.

Gel Formation. Gels were formed by mixing 0.1 mL of 4.1 M NaOH into 3.3 mL of 8 × 10^{-3} M βLg (∼15 wt % crude powder) solution inside a cylindrical glass capsule (final pH 11.2 ± 0.05), heated at 50 °C for 20 min.[11] Caustic-induced gels were chosen because it is convenient to have an initially high ionization degree (fast swelling) and they are easy to handle. The pH 11.2 condition was chosen because it lies above the pK_a of the lysine and tyrosine residues, enabling the gel to swell significantly,[23] while avoiding too high a concentration of added Na$^+$ (0.12 M).

Hydrogen Ion Equilibrium. The pH of a βLg solution after the addition of small volumes of 4 M NaOH was measured with a pH meter (Mettler Toledo, MP220), calibrated with standard buffers at pH 4, 7, 9.21, 10, and 13. Eight different titration series with protein concentrations lying between 2 × 10^{-3} and 5 × 10^{-3} M βLg were performed. The accuracy and measurement repeatability of the high pH buffers was approximately ±0.05 pH units. The optimization of model parameters, for the hydrogen ion equilibrium (eq 25) and for the swelling model (eqs 21 and 22), was performed by minimizing the sum of the square of the residuals using Matlab 6.5.

Determination of Swelling Equilibrium. Initially cylindrical βLg gels were cut into disks, 10.8 mm in diameter and 1−2 mm in height (between 0.2 and 0.5 g of gel), and immersed in NaOH solution at the desired pH and salt concentration. The swollen gel was removed from the solution, dried off with tissue paper, weighed, and replaced in the solution. Measurements were performed until a constant weight was reached. Due to the lack of a buffer in the solution, which would affect the ionic strength, the alkalinity was monitored with a pH meter. For those conditions where the pH of the gel (11.2) was higher than the pH of the solution, the swelling agent was renewed several times until the hydroxide concentration at equilibrium reached the desired value. Swelling equilibrium was always obtained in less than 5 days. The solutions were kept at room temperature, 19 ± 2 °C. Preliminary experiments at different temperatures (<40 °C) showed that the temperature had a negligible effect on the swelling equilibrium. Experiments were performed in duplicate. The volumetric swelling ratio, Q, was determined gravimetrically, assuming the additivity of volumes through the following equation

$$Q = \frac{1}{\phi_{2,\mathrm{s}}} = 1 + \frac{\upsilon_{\mathrm{sp},1}}{\upsilon_{\mathrm{sp},2}}\left(\frac{1}{w}\frac{m_{\mathrm{sw}}}{m_{\mathrm{r}}} - 1\right) \tag{26}$$

where $\upsilon_{\mathrm{sp},1}$ is the specific volume of the solvent (water), w is the weight fraction of βLg in the relaxed gel, i.e., 0.141 g βLg g^{-1} sol, m_{r} is the mass of the gel in the relaxed state, and m_{sw} is the mass at swelling equilibrium. If gels eventually started to dissolve or became too weak so that they eroded, then m_{eq} was set at the maximum mass value. The polymer volume fraction at the relaxed state, $\phi_{2,\mathrm{r}}$, was found with eq 26 using $m_{\mathrm{sw}} = m_{\mathrm{r}}$. The value of Q at the relaxed state is approximately 9.1; lower Q values imply that the gels shrank.

Results and Discussion

Protein Charge at Alkaline pH. Equation 25 was fitted to the experimental data using the N_k values from the βLg amino acid sequence (1BSY, Protein Data Bank), while the basicity constants were optimized from typical values. Figure 1 shows the experimental results from several titration series at different protein concentrations and the calculated hydrogen ion equilibrium curve using the best-fitted parameters in Table 1. Large uncertainties were found in the experimental values close to pH 13 and were discarded from the optimization process. Therefore, the pK_a values of the Ser and Thr residues in Table 1 are only rough approximations. For a satisfactory curve fit, the N_k values for the Tyr and Lys residues were reduced by one from the number found in the amino acid sequence. Nozaki et al.[39] reported similar titration behavior. Their data, obtained at an ionic strength of 0.15 M, are shown in Figure 1 for comparison.

Figure 2 shows that the behavior of the net number of negative charges per protein, $N_\pm$, follows that of the hydrogen

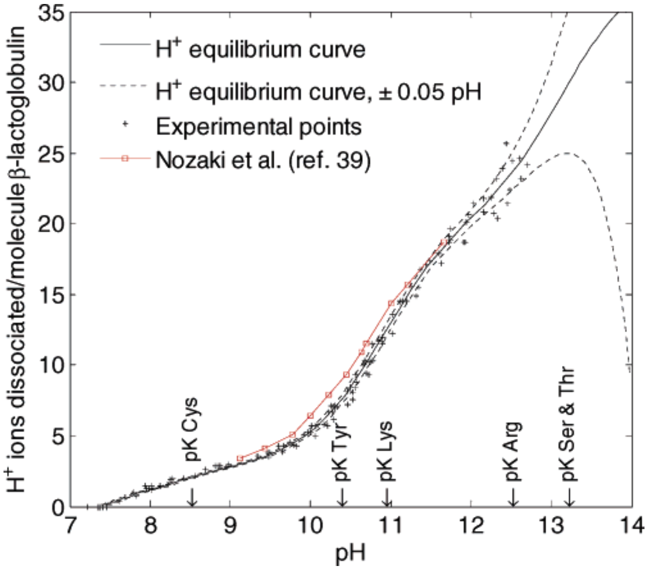

Figure 1. Hydrogen ion equilibrium curve for β-lactoglobulin at 20 °C in deionized water. The hydrogen ion equilibrium curve is calculated using eq 25 with the parameters given in Table 1. Dashed lines show the confidence margin for $\pm$0.05 pH units accuracy in the measurements, for a 4×10^{-3} M βLg solution. Data reported by Nozaki et al.[39] at 0.15 M ionic strength are shown for comparison. The calculated pK_a's for the different ionizable residues are marked with arrows.

ion equilibrium curve, while the total number of charges varies between 37 and 45. Equation 9 assumes that there is perfect compensation of the fixed charges in the protein, as suggested by experiments with synthetic polyampholyte gels.[42−44] It should be noted that the swelling model developed by Şen and Güven[34] for simple amphiphilic hydrogels, as well as being an extension of the BPP model, considers the total and not the net number of charges in the polymer chain. Hence, the predicted swelling behavior with this type of model is only reasonable when the acid and base equilibria are far apart, i.e., when positive and negative charges are not present simultaneously in the polymer chain.

The simple treatment of the ionic contribution to the chemical potentials shown in eq 13 is not satisfactory when counterion condensation occurs over the proteins. When condensation occurs, the immobile counterions do not contribute to the swelling pressure, and an effective charge fraction should be used.[45] According to the classic Manning theory,[46,47] counterion condensation for 1:1 electrolytes occurs when

$$\frac{l_B}{d} > 1 \tag{27}$$

where l_B is the Bjerrun length (7.1 Å for water at 25 °C) and d is the average polyelectrolyte charge spacing projected onto the contour axis.[47] The length d is defined as $d = L/N_{\pm}$, where L is the end-to-end distance of the chain in the state of maximum extension. The estimation of d is nontrivial for globular proteins, as its size depends on the denatured state. A complete stretching of the polypeptide chain is not feasible, as even at pH > 12 there is still a significant amount of secondary structure in βLg ($\sim$20% β-sheets).[48,49] From intrinsic viscosity measurements a maximum stretched length of $\sim$400 Å is reported for βLg in formic acid,[50] and using data in 6 M GuHCl and nonreducing conditions,[51] a maximum length of $\sim$300 Å can be estimated in aqueous solution. The presence of a net charge on a polyampholyte chain has been suggested to deform the native globule into a "necklace" chain of beads and strings.[52] The total length of the elongated necklace chain can be estimated with eq 28,[52] where b is the length of the monomer, $\sim$3.7 Å for an amino acid, and $N_{\beta\text{Lg}}$ is the number of residues in βLg, 162

$$L \approx bN_{\pm}\sqrt{\frac{N_{\beta\text{Lg}}}{N_T}} \tag{28}$$

At pH 7, 10, 11, 12, and 13 we found L values of 76, 105, 166, 230, and 280 Å, and d is calculated to be 7.0, 6.9, 7.3, 7.7, and 7.5 Å, respectively. Therefore, since the calculated values of d are equal or greater than l_B, counterion condensation is unlikely to occur (eq 27), and for practical purposes, the real charge of the protein can be considered to be equal to $N_{\pm}$.

Finally, eqs 6 and 7 assume that the degree of ionization of the amino acids is not affected by the ionic strength. The pK_a shift to lower values when the ionic strength[39] is increased is less than 0.2 pH units. Although this shift has been observed in swelling experiments,[53] this correction is omitted here for simplicity.

Swelling of βLg Gels at Different pH's and Ionic Strengths. Swelling studies on simple polyelectrolyte hydrogels[54,55] have shown that the plot of Q with pH follows the hydrogen ion equilibrium curve of the polymer. Hence, as the number of fixed charges increases due to the ionization of the different groups, Q also increases. However, it has been reported that in the swelling of some amphiphilic polymers at high alkaline pH (>10), Q can decrease markedly despite $N_{\pm}$, remaining practically unchanged.[56,57] It has been suggested that at high NaOH concentrations, the sodium ions of the base can shield the fixed negative charges in the polymer, hindering swelling. This behavior is well-known when the electrolyte is a salt, and it is termed the polyelectrolyte screening effect.[31]

If it is assumed that NaOH does not hinder swelling at high alkaline pH (Figure 3, dashed lines), where the ionic strength is only due to the presence of the salt, then the calculated profile of Q follows the one shown for the hydrogen ion equilibrium curve (Figure 1). However, if the NaOH is included in the total ionic strength (eq 29, for 1:1 electrolytes), then the model predicts that Q will decrease at high pH (Figure 3, continuous lines). Figure 4 shows the experimental results for gels swollen at seven different NaCl concentrations. It can be observed that there is a noticeable polyelectrolyte screening effect for the base cations as Q diminishes at high pH ($\sim$14)

$$I = c_s^* + c_{OH}^* \tag{29}$$

The expected increase in Q above pH 13 due to the ionization of Ser and Thr residues (Figure 3, dashed lines) is masked by the screening effect at these NaOH concentrations, and therefore, the accurate determination of the pK_a values of those residues is not critical for the model. The result of this is only one large change in $N_{\pm}$, between pH 10 and 12, which should then be observed in the Q profile (Figure 4). However, it is clearly evident that Q rises sharply above pH 12, especially at low NaCl concentrations. Under these conditions, reliable Q values are difficult to obtain experimentally, as dissolution of the gel starts to occur and swelling equilibrium cannot be reached. The failure of the present model between pH 11.5 and 13.5 and at low NaCl concentrations will be discussed later. The experimental Q values obtained under these conditions have not been used in the subsequent optimization of the model parameters.

Table 1. Best-Fit Parameters for the βLg Hydrogen Ion Equilibrium Curve, Eq 25[a]

	amino acid						
	Asp, Glu, and α-COOH	His	Cys and α-NH_2	Tyr	Lys	Arg	Ser and Thr
N_k	28	2	2	3 (4)	14 (15)	3	15
$K_{b,k}$	1×10^{10}	1×10^8	3×10^5	4×10^3	1.1×10^3	30	6
pK_a	4	6	8.5	10.4	10.95	12.52	13.22

[a] The values in brackets are from the βLg amino acid sequence (1BSY, Protein Data Bank).

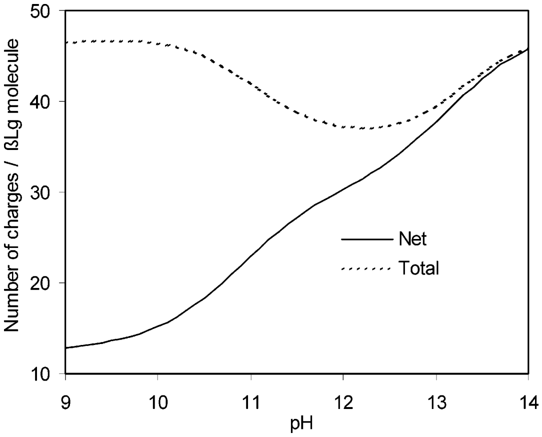

Figure 2. Effect of pH on the net number of active negative charges, $N_\pm$ (continuous line, eq 9), and total number of charges, N_T (dashed line, eq 10) per βLg molecule calculated using the parameters in Table 1.

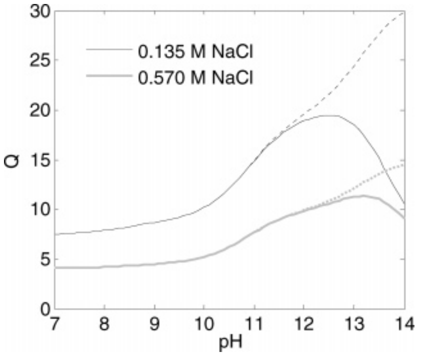

Figure 3. Effect of pH on the simulated equilibrium swelling degree (eq 17) considering (i) NaOH contributes to the global screening effect (continuous lines, $I = c_s^* + c_{OH}^*$ (eq 29)) and (ii) no contribution (dashed lines, $I = c_s^*$).

We have used eq 13 to model the ionic contribution to the chemical potentials because both salt limits apply: For the experiments under 0.06 M NaCl, $c_\pm \geq I$, while above 0.135 M NaCl, $c_\pm < I$. Therefore, the high-salt limit regime (eq 15) is not fully applicable across the whole range of c_s^* values studied. As described in the Theory section, the model has three adjustable parameters, ν_e and the Flory–Huggins parameters χ_0 and χ_1. Equation 21 has been used to calculate the solvent–protein interaction parameters (Figure 5), while ν_e has been determined using eq 22 (Figure 6). Figure 5 also shows the experimental points at high pH and low NaCl concentrations that were not used for the optimization of the model parameters, as discussed previously, and which fall below the best-fit line for eq 21. The overall agreement of eq 17 is shown in Figure 4. The model parameters obtained were: $\chi_0 = 0.513$, $\chi_1 = 0.31$, and $\nu_e = 270$ mol m^{-3}; while the other parameters used were: $\phi_{2,r} = 0.11$, $\upsilon_{sp,2} = 0.75 \times 10^{-3}$ m^3 kg^{-1} (ref 49), and $\upsilon_{m,1} = 1.8 \times 10^{-5}$ m^3 mol^{-1}.

The thermodynamic swelling model proposed has the same limitations as the original BPP model, although it is capable of reproducing the trends observed experimentally. The present model could be improved following the work done in polyelectrolyte polymers,[58] taking into account the activities of the chemical compounds, the excluded volume of the ions, and excess free energy corrections.

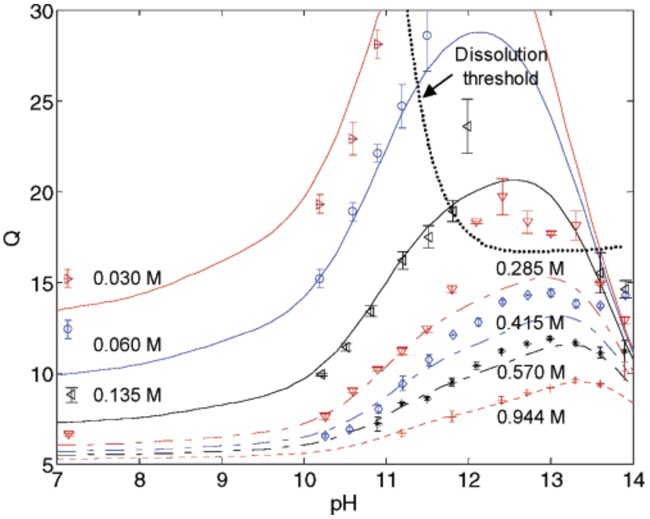

Figure 4. Effect of pH on the equilibrium swelling degree in βLg gels at different NaCl concentrations at 19 °C. Lines are calculated with eq 17 as described in the text. The dotted line shows the observed dissolution threshold. Experimental points on the top-right side of the dissolution threshold line are for gels that started to dissolve before reaching swelling equilibrium. Error bars show standard error.

Scaling Laws for Swelling. Rubinstein et al.[28] have reported the scaling laws for the swelling of polyelectrolyte gels with the mobile electrolyte concentration

$$Q \sim (c_s^*)^{-m}(N_\pm)^n \tag{30}$$

The scaling factor m depends on the salt concentration. In the low-salt limit, when the concentration of counterions in the gel is much higher than that of the mobile electrolyte, $c_\pm \gg c_s^*$ (eq 14), m tends to zero. In the high-salt limit, $c_\pm \ll c_s^*$ (eq 15), there are two possible regimes. In the weak screening regime, the Debye–Hückel screening length (κ^{-1}) is larger than the mesh size of the gel (ξ), while in the strong screening regime,[35] $\kappa^{-1} < \xi$. κ^{-1} inside the gel was below 20 Å for all gels studied, while ξ in βLg gels is about 10 times this value.[22,59] In the high-salt limit with strong screening, the scaling factor m has been shown to be 0.6.[28,37] In the presence of a large excess of salt, typically when $\kappa^{-1} < l_B$, the strong screening inside the gel makes the gel behave as a neutral polymer,[37] and the swelling degree is given by the c* theorem.[60] As Q is independent of the salt concentration in neutral gels, m decreases to zero at high c_s^* values. However, it has been observed in balanced or weakly charged polyampholyte gels that Q increases at high c_s^* values (~1 M),[43,61] yielding a negative value of m. This behavior was not observed in the βLg gels studied, as Q was found to be constant at 6 ± 0.1 above 1 M NaCl (Figure

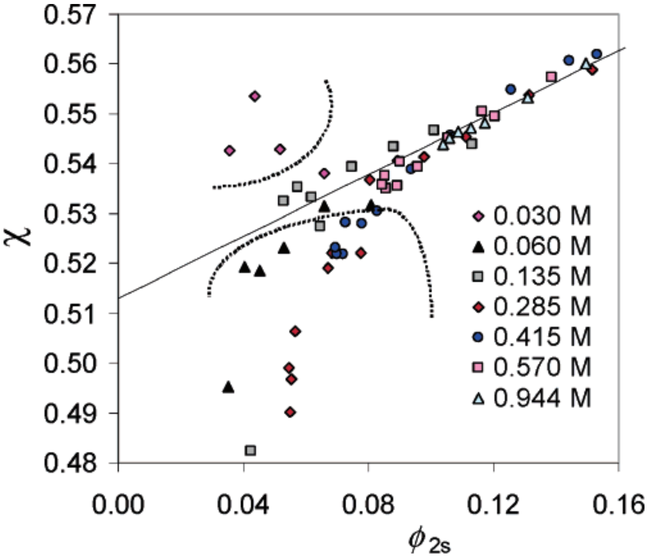

Figure 5. Determination of the Flory−Huggins parameters, eq 21. The solid line shows the best-fit linear trend with parameters $\chi_0 = 0.513$ and $\chi_1 = 0.31$. The symbols denote NaCl concentration. Data delimited by the dashed lines were not used for the determination of χ as explained in the text.

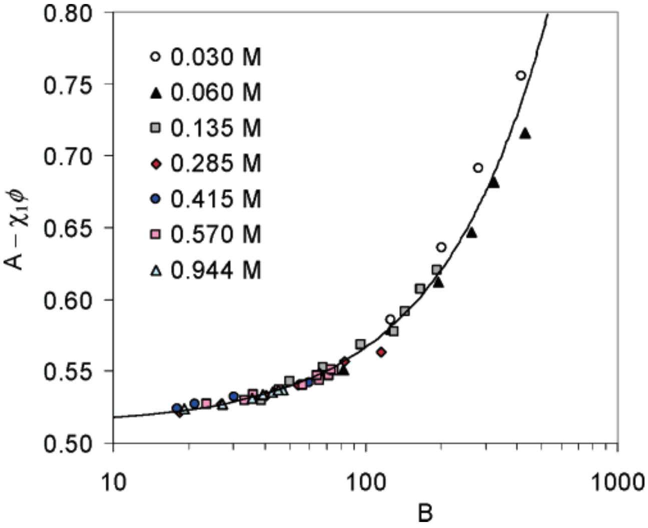

Figure 6. Determination of the cross-linking density, eq 22. Best-fit value: $\nu_e = 270$ mol m^{-3}. The symbols denote NaCl concentration.

7). The large difference in numbers of negative to positive charges in the chain at alkaline pH makes the protein gel behave like a polyelectrolyte gel.[43,52]

The transition from $m = 0$ at very low c_s^* values ($<10^{-4}$ M), followed by $m \leq 0.6$ at medium c_s^* values ($10^{-1}-10^{-3}$ M), and again to $m = 0$ at very high c_s^* values ($\sim 10^0$ M) has been reported often in polymer gels.[54,62,63] The maximum m values observed in polymers are usually below the predicted scaling factor of 0.6,[28,37] probably due to the transitions at low and high c_s^* values that reduce the apparent value of m.

Although there is no reason to believe a priori that the scaling laws for polyelectrolyte polymer gels should be applicable also for concentrated globular protein gels, Figure 7 shows that the experimental profile of Q with c_s^* at pH 11.2 is similar to the one discussed previously for polyelectrolyte gels. At low and high c_s^* values, the Q values reach a plateau, while between 0.03 and 1 M NaCl Q decreases with increasing c_s^* values, described by a scaling factor of 0.45 ± 0.02. In addition, it is observed that the type of the alkali cation (Li, Na, or K) does not significantly affect the degree of swelling, as in polymer gels.[30,57] The scaling eq 30 is also observed to be applicable at other pH values, as shown in Figure 8. The lines shown in Figure 8 are calculated with the swelling model presented previously (eq 17). Figure 9 shows the calculated m values from the experiments and from the model at different pH's. Good agreement is observed below pH 12, where an average value of 0.47 is found with the model in the alkaline range (Figure 9, dashed line). Above pH 12, the model predicts a decrease of m as the NaOH concentration starts to be of the same order as the NaCl concentration, and the screening effect due to the base cation is enhanced. However, the experimental m values do not show a decrease above pH 12. The accuracy of m under these conditions is poor because swelling experiments at low c_s^* values feature rapid dissolution. In general however, an m value higher than 0.47 is observed rather than a decrease.

The calculated scaling factor for $N_{\pm}$, n from eq 30, decreases slightly with increasing c_s^* values, with an average value of 1.04 ± 0.07 in the range of 0.03−0.415 M NaCl and below pH 12. This value is again lower than the theoretical scaling factor for the ionization degree in synthetic polyelectrolytes gels of 1.2.[37] Skouri et al.[37] argued that the ionization degree of the

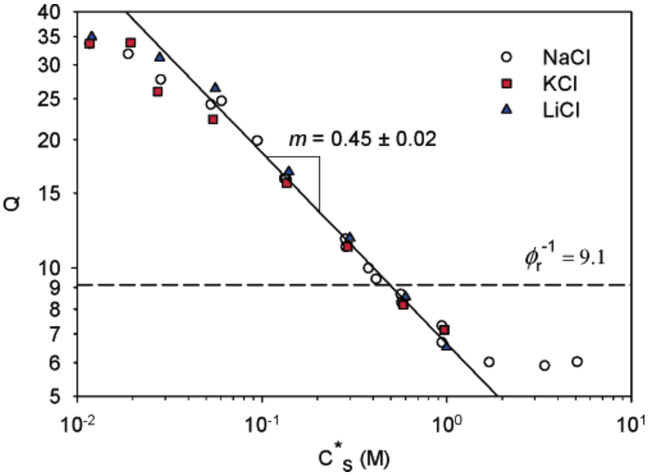

Figure 7. Effect of c_s^* on the equilibrium swelling ratio for NaCl, KCl, and LiCl electrolytes at pH 11.2. The line shows the experimental scaling law found (eq 30). The dashed line shows the Q value of the gels in the relaxed state.

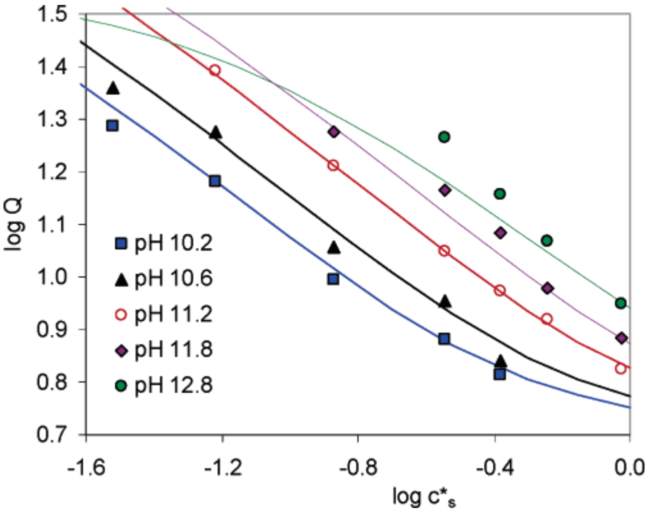

Figure 8. Effect of c_s^* on the equilibrium swelling ratio at different pH's. Symbols are experimental values. The lines are calculated from eq 17.

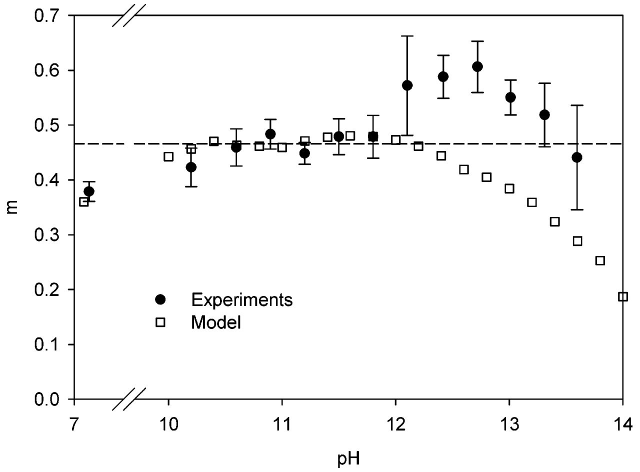

Figure 9. Scaling factor *m* (eq 30) at different pH's in the range 0.05 < c_s^* < 1 M NaCl. The dashed line shows the average value of *m* at pH 10−12, 0.47 ± 0.02.

polymer and the Debye length (as a measure of the ionic strength) should have the same scaling factor, i.e., 1.2. In the present study we also found that the scaling factors are similar, albeit lower than 1.2, at 0.94 ± 0.05 for κ^{-1}, which is twice the value of m, and 1.04 ± 0.07 for $N_{\pm}$.

Dissolution Threshold. We have shown that a simple swelling model derived for polyelectrolyte gels, adapted to take into account the several ionization equilibria involved in a protein, yields satisfactory results for βLg gels, which are commonly used as a model for globular protein gels. However, the proposed model greatly underpredicts Q when the gels swell above pH ∼12 and <1 M NaCl (Figure 4). The failure of the model under such conditions is also observed when calculating the best-fit χ value (Figure 5) and the scaling factor m (Figure 9). The large increase in Q at pH's higher than 12 cannot be modeled with the proposed model if χ and ν_e are considered to be constant, as the Q profile will follow the behavior of the hydrogen ion curve (Figures 1−3). Either a decrease of χ (an improvement in the solvent quality) or a decrease of ν_e (disruption of entanglements or cross-links in the gel) will be needed for the model to agree with the experimental data.

The failure of the model is of great interest as the conditions where the model underpredicts Q are close to those when the gels start to be dissolved. For example, Mercadé-Prieto and Chen[11] reported that for whey protein concentrate (WPC) gels, the dissolution rate observed below pH ∼12 was similar to those for deionized water, while for βLg gels exposed to a pH of 12.8, the dissolution rate became very small when using >0.35 M NaCl solutions.[16] In the present study, no visual degradation of the gels was observed at pH 12.4−13.3 in 0.415 M NaCl before reaching swelling equilibrium after 1 day, but it was clearly noticeable in 0.285 M NaCl after 12 h. These observations clearly suggest that the onset of dissolution is related to exceeding a minimum pH, probably approximately 11.5−12, and to exceeding a minimum swelling degree (say $Q > 17$). Figure 4 shows a suggested locus for the dissolution threshold. Gels at pH < 11 and 0.03 M NaCl (high Q but low pH) or at pH ∼13 and 0.944 M NaCl (low Q but high pH) were stable for days without any sign of dissolution. Above the dissolution threshold, dissolution was observed before the swelling equilibrium was reached. As a starting point for future work on the concept of a dissolution threshold, we suggest that the effect of the pH may be related to the disruption of weak interactions between βLg covalently cross-linked clusters,[64] which would modify the parameters χ or ν_e. However, the existence of a minimum in Q may be related to a transition below the entanglement concentration, c_e, enabling those cross-linked clusters to disentangle easily.[16,65] For example, c_e is approximately 5−10 times higher than the overlapping concentration (c^*) in salt solutions;[27] c^* is about 11 g βLg L^{-1}.[59] Hence, c_e is on the order of 55−110 g βLg L^{-1}, in agreement with the concentration at the minimum Q for dissolution to occur, $15 < Q < 20$, 68 and 92 g βLg L^{-1}, respectively.

Conclusions

Although the variety of different amino acids in a protein allows it to have much more complex configurations than a typical synthetic polymer, as shown by the fact that approximately 20% of the residues are still not in contact with the solvent at a highly alkaline pH,[49] the simple swelling equations developed for polyelectrolyte gels seem to be applicable to βLg gels. A simple modification has been applied to those models to account for the different ionizable residues in a protein (eqs 3−7). Moreover, it is of greater interest, particularly in the area of dissolution of protein gels, that the model fails under certain conditions of pH and c^*_s. Unlike most of the synthetic polymers used in swelling studies, βLg gels eventually dissolve in alkali because they do not form a perfectly covalently cross-linked network. Our results suggest that there is a dissolution threshold, below which gels swell but do not dissolve. The threshold is characterized by a minimum pH of 11.5−12 and by a minimum extent of swelling ($Q \approx 15-20$). More research is needed to confirm and understand the existence of a minimum pH and Q for dissolution to occur.

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