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Intrinsic fluorescence investigation on the change in conformation of cross-linked gelatin gel during volume phase transition

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Abstract

Gelatin gel cross-linked with glutaraldehyde was prepared. Its volume is sensitive to the acetone–water composition. Near 50% acetone content, a discontinuous macroscopic volume phase transition (VPT) occurred. The gel was found to emit intrinsic blue fluorescence at 415 nm, which is attributed to the formation of dimeric species of tyrosine (bityrosine) in the gelatin network. The ratio of intensity of bityrosine to that of tyrosine monomer, I_B/I_M, was used to examine the change in conformation of gelatin during volume phase transition (VPT). With the increasing acetone concentration, I_B/I_M continuously increased till VPT, and then dropped little, revealing a high yield of bityrosine species, which is considered to stem from the closer contact of inter-coil, inter-helix, intra-helix and coil-helix in the collapsed network. The continuous I_B/I_M change suggests the non-discontinuous volume phase transition of gel occurring in microscale. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Gelatin; Volume phase transition; Intrinsic fluorescence

1. Introduction

Gelatin is a denatured collagen, the most abundant protein in hides, skins, bones and connective tissue of mammals [1]. It is used in a variety of gel applications including photography, drug delivery, holography, micro-encapsulation and food preparation. In the past decades, much attention has been paid to the gel properties as well as the structural characteristics of gelatin chains in solution [2–7]. However, the structure of chemically cross-linked gelatin gels has been studied little compared with thermo-reversible gelatin gel. A decade ago, Tanaka [8] found that the volume of chemically cross-linked gelatin gel varied in responding to the compositions of water–acetone mixture; at a certain acetone content, the gel underwent a discontinuous volume phase transition. Tanaka's work is only confined to the macroscopic visualization of volume change. It is well known that the ordering of gelatin networks is characteristic of the amino acid sequence, which contains a well conserved glycine–proline–hydroxyproline triad throughout the whole protein [9]. Recently, Watanabe [10] investigated the effect of cross-linking degree on the content of triple helix in cross-linked gelatin using pyrene labeling fluorescence depolarization method. It was reported that a small amount of cross-link promoted the regeneration of the collagen triple helix. Although extrinsic fluorophores can make up the insensitivity of natural fluorescence of macromolecules in some cases, an extrinsic fluorophore usually has huge aromatic rings cross-linked to the protein, which inevitably results in the alteration in microenvironment and local conformation. In general, protein contains three amino acid residues, which contribute to their naturally occurring or intrinsic ultraviolet fluorescence: tryptophan, tyrosine, and phenylalanine. Tyrosine is highly fluorescent in solution, but its emission is generally weaker in protein. Denaturation of proteins can result in an enhanced tyrosine emission [11].

Gelatin is a derivative of collagen. So far as we know, few researchers have dealt with its fluorescence characteristics and studies of its conformation by its intrinsic fluorescence have not been reported.

Recently, we accidentally found that the fluorescence of gelatin gel was sensitive to acetone–water composition, moreover, the ratio of intensity of blue fluorescence at 415 nm (attributed to the dimerization of tyrosine in gelatin, bityrosine) to that of tyrosine monomer is dependent upon gelatin volume. In this work, we did exploit the intrinsic fluorescence to initially probe the change in conformation

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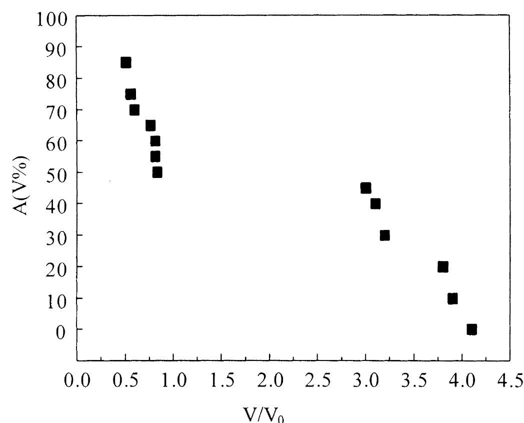


Fig. 1. Volume swelling ratio vs. acetone concentration.

of gelatin gel during the volume phase transition induced by a change in acetone–water composition.

2. Experimental

2.1. Materials

Gelatin (type B, extracted from bovine skin) was purchased from Sigma Chemical Co. St. Louis, MO, USA. Glutaraldehyde and acetone were of analytical grade. L-tyrosine (analytical grade) was kindly provided by Tianjin Hygienic Materials Factory.

2.2. Preparation of gelatin gels

To doubly distilled and deionized water, 5% gelatin was added and dissolved at 40°C. Glutaraldehyde with 2 wt% of gelatin was added slowly under stirring as the cross-linking agent. The mixture was poured into a plastic culture dish and cured at 50°C in a vacuum oven for film formation.

2.3. Fluorescence measurements

The gelatin gels were swollen in a series of acetone–water solutions until they reached equilibrium swelling.

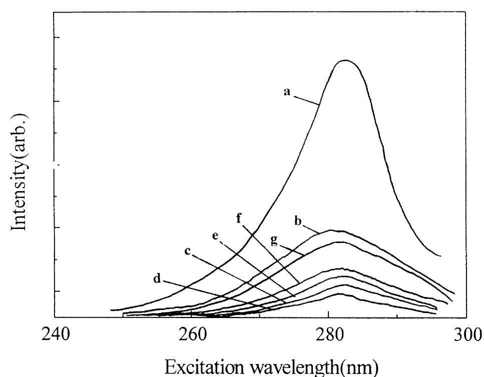


Fig. 2. The excitation spectra measured at various acetone contents. (a) 0%; (b) 10%; (c) 30%; (d) 50%; (e) 60%; (f) 70%; (g) 85%.

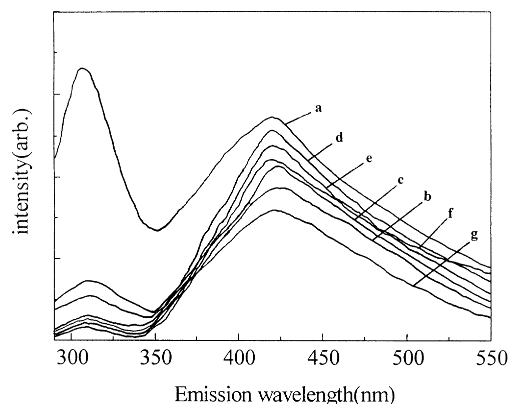


Fig. 3. The fluorescence emission spectra measured at various acetone contents. (a) 0%; (b) 10%; (c) 30%; (d) 50%; (e) 60%; (f) 70%; (g) 85%.

Then the gels were cut into a suitable size and put into a quartz cell containing an acetone–water mixture of composition identical to that used for gel swelling. The steady-state fluorescence spectra of gels were recorded with the front-face configuration on a SPEX FL212 Spectrofluorometer in the direction of 22.5° to the excitation light. The excitation wave length was set at 280 nm and slit width was chosen to be 2.58 nm/2.58 nm.

3. Results and discussion

3.1. Macroscopic measurement of the variation in volume of gelatin gel

The dependence of equilibrium swelling degree of gelatin gels, $V/V_0 = (d/d_0)^3$, where, d and d_0 are the initial and final diameters of gel, respectively, upon the acetone volume content (V%) is shown in Fig. 1. A discontinuous VPT is observed at 50%, which demonstrates a VPT of our samples.

3.2. Intrinsic fluorescence characterization of gelatin gel during VPT

The excitation spectra monitored at 305 nm are displayed in Fig. 2. The emission spectra for various acetone concentrations are exhibited in Fig. 3, in which two emission peaks at 305 and 415 nm appear. It is well known that the fluorescence of protein is originated mainly from tyrosine, tryptophan and phenylalanine. Besides gelatin contains ca. 1% tyrosine, 2–3% phenylalanine, and no tryptophan [12]. Phenylalanine is not excited in most cases and its quantum yield of phenylalanine in protein is rather low, so that the emission from this residue can be ignored. Hence, the fluorescence is exclusively resulted from tyrosine in the gelatin gel. As shown in the figure, the emission band at 305 nm typically stems from tyrosine residue [11]. However, the peak centered around 415 nm is somewhat surprising. At first we suspected it resulted from an impurity, thus we recorded the emission spectra of original gelatin powder and water used with the same condition. The results are

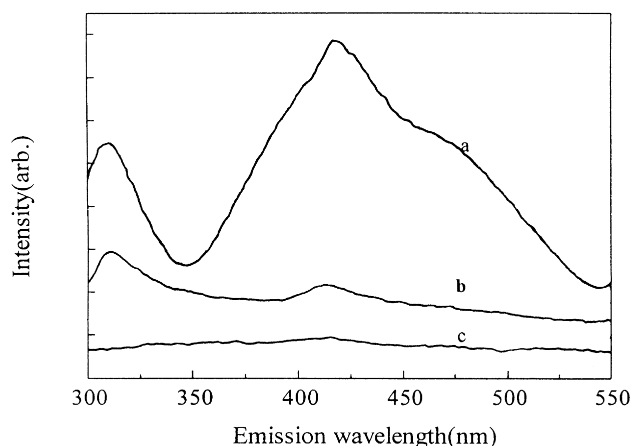
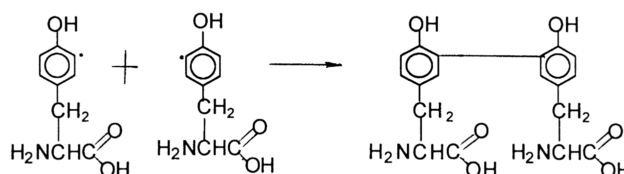


Fig. 4. The fluorescence emission spectra of: (a) original gelatin powder; (b) dilute hydrochloric acid solution of tyrosine; and (c) water.

shown in Fig. 4. For gelatin powder, two maxima at 305 and 415 nm are present, while water displays no emission peak at these two wavelengths. Thus, it is reasonable to eliminate the possibility that the emission peak at 415 nm stems from the impurity in water. Then the unexpected peak may arise from the impurity in gelatin or a certain derivative species of tyrosine itself. In view of that, we checked the fluorescence of a dilute hydrochloric acid solution of tyrosine (10 mg of L-tyrosine was dissolved in 100 g hydrochloric acid). The emission spectrum is shown in Fig. 4. Analogously, two peaks appear at 305 and 415 nm, respectively. This indicates that the emission peak at 415 nm is attributed to the derivative of tyrosine.

In the 1960s, Lehrer and Fasman [13] systematically investigated the blue fluorescence of solutions of poly-L-tyrosine, a copolymer of tyrosine and L-tyrosine monomer. His modeling experiments demonstrated that the emission band at 415 nm was from photodimerization of tyrosine. Tyrosine dimeric product was produced via the combination of transient phenoxy radicals formed by photoelectron ejection from the phenol. The formation mechanism of dimeric species-bityrosine, is described as follows [14]:



In gelatin gel, this phenomenon may occur if the steric arrangement of tyrosine residue allows two tyrosine units to be in close proximity. A variation in volume of gelatin will undoubtedly influence the encounter probability of tyrosine residues, leading to a change of bityrosine yield. In Lehrer's experiment, the fluorescence of poly-L-tyrosine was shifted several nanometers to longer wavelength when the sample was transferred from base to dimethyl sulfoxide. Comparatively, in our case, no wavelength shift is observed at various acetone contents, while the fluorescence intensity varies with the change of acetone concentration. Considering a possible effect of acetone and the change in polarity of solvent on the fluorescence intensity, all the spectra were calibrated by subtracting the fluorescence of corresponding solution from that of gelatin hydrogel sample. Fig. 5 shows the ratio of I_B/I_M against acetone content, where I_B is the bityrosine emission intensity at 415 nm, and I_M is the intensity of tyrosine monomer at 305 nm. In the absence of acetone, the gel is highly swollen, I_B/I_M is about 0.79; with the increase of acetone, I_B/I_M increases continuously. At 50%, i.e. approaching VPT, I_B/I_M reaches a maximum 14.3. On further increase in acetone concentration, I_B/I_M drops slightly. The change in I_B/I_M should reflect the variation in the microenvironment of tyrosine and conformation of gelatin gel. For chemically cross-linked gelatin gel, its network is composed of chemical cross-linked sites, triple-helices and end random coils connecting triple-helices [15]. As gelatin gel is swollen, owing to a large amount of water molecules in networks, the distance between different segments is expanded. In addition, in amino acid sequences, there are only a few amounts of tyrosine residues. Thus, in highly swollen gelatin gel, the encounter probability of tyrosine is lowered. For comparison, the I_B/I_M of 0.01% gelatin aqueous solution is merely 0.038, factor of 20 smaller than that of swollen gel, suggesting a rather low yield of bityrosine. A rational explanation is that in extremely dilute solution, triple-helices are destroyed to produce random coils, which are isolated by a large amount of free water molecules, making it very hard for tyrosine residues to encounter. Therefore, we think the production of bityrosine in highly swollen gel is mainly from the triple-helices in the network. Triple-helices are regarded as rigid rods of approximately 15 Å in diameter and 3000 Å in length. The distance between the rods cannot be more than several Angstroms [16]. The short distance may allow contacts of tyrosine residues distributing in the amino acid sequences. With the increase of acetone concentration, I_B/I_M increases

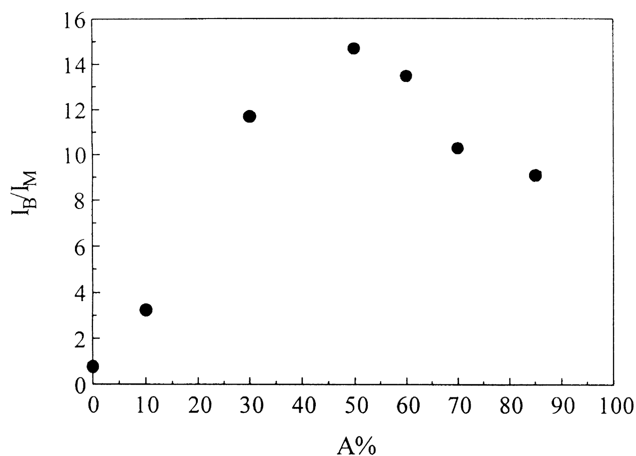


Fig. 5. The dependence of I_B/I_M on acetone contents.

and reaches a maximum at VPT. At VPT, the collapse of volume expels most of water in the network, and consequently, the random coils in swollen state are brought to contact one another, ultimately form dense globule-like structure. The sharp reduction in volume of gel can also result in the dense stack of triple-helices. From Fig. 5, one also finds that I_B/I_M slightly decreases above VPT. A reasonable explanation is that with the increase in rigidity of molecular chain caused by volume shrinkage, the mobility of chain is markedly lowered, resulting in a decrease in collision probability of tyrosine. Thus, it is proposed that as the volume of gelatin occurs to collapse, the higher yield of bityrosine primarily originates from the closer contact of inter-coil, inter-helices, coil-helix, intra-helix, the mobility helix, the mobility of chain may be responsible for the collision probability of tyrosine as well. It should be stressed that I_B/I_M shows no discontinuous change over the whole range of acetone concentration. The result shown in Fig. 1 reflects the macroscopic volume change of gel, while fluorescence spectra determine microscopic local variation of chain by probe molecule. From the above analyses, we argue that a discontinuous volume phase transition of gel occurs only in macroscale; in microscale, it is actually a continuous change. A similar result has been elucidated by Wu's light scattering studies of *N*-isopropylacrylamide microgel [17].

4. Conclusions

Gelatin gel cross-linked by glutaraldehyde shows a discontinuous macroscopic volume phase transition near 50% acetone concentration. The intrinsic fluorescence of tyrosine residues in gelatin can be used as a probe to examine the variation in the conformation of gelatin gel. The ratio of fluorescence intensity of bityrosine to that of tyrosine monomer varies with the content of acetone. In swollen state, I_B/I_M is lower; upon increasing acetone content, that is along with the decrease of volume, I_B/I_M continuously increases till VPT point. This implies closer encounter of inter-coil, inter-helix, coil-helix, intra-helix in the collapsed network. The continuous I_B/I_M change suggests that the volume phase transition of gel occurring microscopically is non-discontinuous.

Acknowledgements

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References

[1] Ward AG, Courts A. The science and technology of gelatin. London: Academic Press, 1977.
[2] Herning T, Djabourov M, Leblond J, Takerkart G. Polymer 1991;32:3211.
[3] Bodidar HB, Jena SS. J Chem Phys 1993;98:8970.
[4] Fakirov S, Sarac Z, Anbar T, Boz B, Bahar I, Evstatiev M, Apostolov AA, Mark JE, Kloczkowski A. Colloid Polym Sci 1997;275:307.
[5] Traub W, Piez KA. Adv Protein Chem 1971;25:243.
[6] Djabourov M. Contemp Phys 1988;29:273.
[7] Prystupa DA, Donald AM. Polym Gels Networks 1996;4:87.
[8] Amiya T, Tanaka T. Macromolecules 1987;20:1162.
[9] Fischer KL, Tirrell DA. J Appl Polym Sci 1998;68:281.
[10] Watanabe K. Macromolecules 1997;30:7910.
[11] Lakowicz JR. Principles of fluorescence spectroscopy. New York–London: Plenum Press, 1983 (342p.).
[12] Young HH. In: Herman FM, Norbert MB, Charles GO, Georg M, Jacqueline I, editors. Gelatin in encyclopedia of polymer science and engineering, vol. 7. New York: Interscience, 1967. p. 446.
[13] Lehrer SS, Fasman GD. Biochemistry 1976;6:757.
[14] Anderson SO. Biochim Biophys Acta 1963;69:249.
[15] Oikawa H, Nakanishi H. Polymer 1993;34:3358.
[16] Hoeve CA. In: Rowland SP, editor. Water in polymers, ACS Symposium SeriesWashington, DC: American Chemical Society, 1980 (p. 135).
[17] Wu C, Zhou S. Macromolecules 1997;30:574.