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Unassociated Molecular Chains in Physically Crosslinked Gellan Gels

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ABSTRACT: It was demonstrated that gellan chains unassociated with the crosslinking domains in the gels are released from gellan gels into the external solution at 10 °C. The concentration of released gellan chains in the external solution was estimated using a phenol-sulfuric acid method. The release of gellan chains was suppressed with increasing concentration of potassium chloride or calcium chloride in the external solution whilst it was enhanced with increasing concentration of tetramethylammonium chloride in the external solution. It was suggested from differential scanning calorimetry that the released gellan chains adopt a double helix conformation at 10 °C. The experimental results indicated that the double helical gellan chains, which can be released from the gels, are unassociated with the crosslinking domains composed of the lateral association of double helices in gellan gels. In conclusion, it was shown that the amount of unassociated gellan chains contained in the physical gel can be determined by measuring the concentration of released gellan chains in the external solution. [doi:10.1295/polymj.PJ2006149]

KEY WORDS Gellan Gum / Physically Crosslinked Gels / Release of Polymer /

Gellan is a microbial polysaccharide and has been utilized in various industrial applications due to its ability to form a thermo-reversible gel in aqueous media. The most readily available form of gellan is that of a deacylated form with a tetrasaccharide repeating unit of (1-3)-β-D-glucose-(1-4)-β-D-glucuronic acid-(1-4)-β-D-glucose-(1-4)-α-L-rhamnose (Figure 1).[1,2] Gellan is often regarded as a model system for investigating the gelation mechanism of helix-forming polysaccharides.

There have been various investigations of the gelation and the conformation change of gellan molecules in aqueous solution.[3–16] The gelation of gellan is remarkably enhanced by the addition of cations in aqueous solutions, since the gellan molecules possess carboxyl groups in the repeating unit.[11,12] Potassium, rubidium and cesium ions are categorized as gel-promoting cations. Potassium and rubidium ions have been shown to interact selectively with gellan as determined by multinuclear NMR measurements.[17,18] Lithium and sodium ions are less effective in promoting gelation. Divalent cations such as calcium and magnesium ions effectively promote gelation, generally inducing gelation at much lower concentrations than monovalent gel-promoting cations.[8,19]

The conformational transition from two single chains to a double helix (coil-helix transition) occurs on cooling the gellan solution, and represents a reversible change. The coil-helix transition temperature increases with increasing concentration of added salt.[9–11] Double helix formations and subsequent lateral association of the double helical molecules may achieve formation of the gellan gel network. It has been reported that gellan gels possess a crosslinking domain composed of associated double helices.[20–22] Lateral association of the double helices is an important factor for the gelation of gellan. It has been known that lateral association of gellan double helices is inhibited by transforming counter ions into tetramethylammonium ions (TMA^+).[7,23–25]

Irrespective of the type of crosslinking, some fraction of polymer remains uncrosslinked to gel network. In terms of the gellan physical gel, it seems reasonable to posit that not all of the gellan chains contribute to formation of the gel network. A fraction of gellan chains is expected to be unassociated with crosslinking domains in gellan gel. To the best of our knowledge, very little work has been published in the literature concerning the determination of the fraction of unassociated gellan chains in gellan gel. This article describes attempt to determine the fraction of unassociated gellan chains present in gels.

EXPERIMENTAL

Materials

Gellan was kindly supplied by San-Ei Gen F. F. I., Inc. (Osaka Japan) and was used without further puri-

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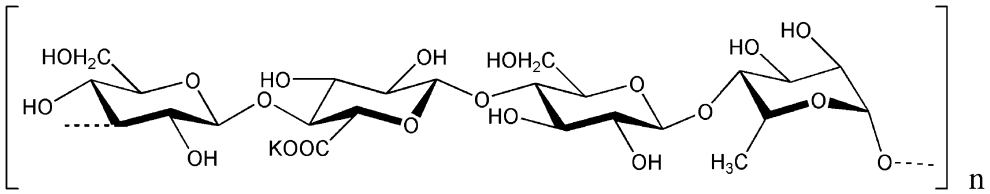

Figure 1. Chemical structure of repeating unit of deacylated gellan (potassium salt).

fication (95% purity). The metal content of dry gellan was analyzed to be Na 0.42%, K 5.03%, Ca 0.37% and Mg 0.09% in weight. Phenol (Wako Pure Chemical Industries Ltd. Japan, 99% purity) and sulfuric acid (Wako Pure Chemical Industries Ltd. Japan, 96% purity) were of reagent grade and used without further purification.

Preparation of Physical Gel

Gellan was dissolved in distilled water at 95 °C. The gellan solutions were kept at 95 °C in a water bath for 4 h to ensure complete dissolution. Hot solutions were poured into cylindrical moulds (stainless, inner diameter of 1 cm, height of 1.5 cm), and the temperature was then lowered to 10 °C at a cooling rate of about 2 °C/min, and maintained at 10 °C for 24 h.

Measurement of Concentration of Released Gellan Chains

To examine the release of gellan molecular chains from the gels, gels were immersed in distilled water or a salt solution. The weight of the external solution and the cylindrical gel was 50 g and about 1 g, respectively. The concentration of released gellan chains into the external solution was measured at appropriate intervals.

The gellan concentration in the external solution was determined using a phenol-sulfuric acid method.[26] The phenol-sulfuric acid method can be used to determine the concentration of reducing sugars such as aldopentoses, aldohexoses and uronic acids in aqueous solutions. Gellan solution (1 mL) and 5% (w/w) phenol aqueous solution (1 mL) were mixed and then concentrated sulfuric acid (5 mL) was added. The solution was then cooled in a water bath at room temperature. The solution has an absorption maximum at 483 nm. The absorbance at 483 nm was measured using an UV-VIS Spectrophotometer UV-mini 1240 (SHIMADZU). The gellan concentration in the external solution, C_{out}, was estimated from the calibration curve of the gellan solution (absorptivity $K = 7.5 \times 103$ g/(g cm)).

Intrinsic Viscosity Measurements

The relative viscosity, η_{rel}, of the gellan solutions was measured at 25 ± 0.01 °C in aqueous solution with 100 mmol kg^{-1} KCl using an Ubbelohde-type viscometer. The flow time of the solvent (100 mmol kg^{-1} KCl aqueous solutions) was 205 s at 25 °C. Specific viscosity, η_{sp}, values were calculated using $\eta_{sp} = \eta_{rel} - 1$. Viscosity data were analyzed using Huggins[27] and Mead-Fuoss[28] plots simultaneously to determine the extrapolation at infinite dilution.

DSC Measurements

Differential scanning calorimetry (DSC) measurements were carried out using a Setaram micro DSC-III calorimeter (Caluire, France). Approximately 800 mg of sample solution was hermetically sealed into a DSC pan and the pan was then accurately weighed. For each sample, a reference pan was filled with distilled water. Sample and reference pans were placed inside the calorimeter, heated to 90 °C, and then maintained at 90 °C for 30 min so that the thermal history could be disregarded. The temperature was then lowered to 5 °C at a rate of 0.5 °C/min.

Mechanical Measurements

Measurements of storage Young's modulus, E', of a cylindrical gellan gel (diameter of 1 cm and height of 1.5 cm) were carried out using a Rheolograph Gel at 25 °C (Toyo Seiki Seisaku-sho Ltd., Tokyo).[29] The frequency of longitudinal vibrations was fixed as 3 Hz and the amplitude was also fixed as 100 μm (strain 0.03) for this apparatus. The gel was immersed in silicone oil to prevent the evaporation of water from the gels and to control the temperature.

RESULTS AND DISCUSSION

Release of Gellan Chains in Distilled Water

Figure 2 shows the concentration of gellan chains in the external solution, C_{out}, as a function of time for initial gellan concentrations, C_g, of 1.5% (w/w), 2.0% (w/w), and 3.0% (w/w). Measurement of C_{out} was carried out at 10 °C, a temperature much lower than the helix-coil transition temperature of gellan (above 30 °C). The C_{out} values increased with time and reached a plateau after *ca.* 50 h for all concentrations. Gellan gels with $C_g \geq 2.0\%$ (w/w) maintained a cylindrical shape after 50 h. The results suggest that gellan chains are released from the gels to the external

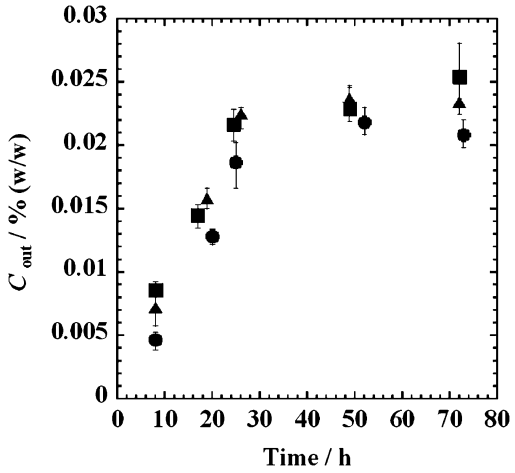

Figure 2. Gellan concentration in the external solution without added salt, C_{out}, as a function of time at 10 °C with initial gellan concentration, C_g, of 1.5% (w/w) (●), 2.0% (w/w) (▲) and 3.0% (w/w) (■).

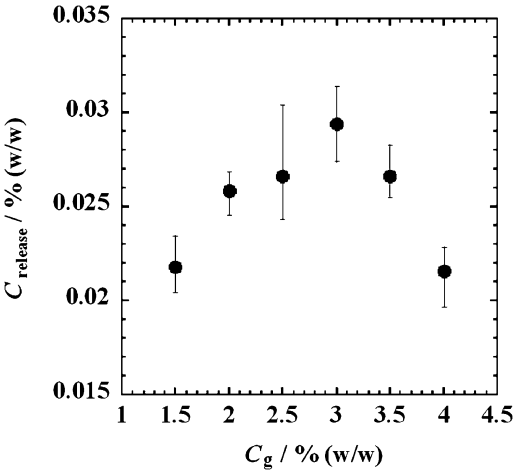

Figure 3. Dependence of the released gellan chains concentration, $C_{release}$, on the initial gellan concentration, C_g, in the external solution without added salt at 10 °C. $C_{release}$ represents the concentration at the plateau after 50 h in Figure 1.

solution. In this study, gellan chains released to the external solution are referred to as "released gellan chains", while the gellan chains remaining in the gels are referred to as "unreleased gellan chains". It is highly probable that the released gellan chains are uncrosslinked to the gel network.

The concentration of gellan chains, reaching a plateau after 50 h as shown in Figure 2, is defined as the concentration of released gellan chains, $C_{release}$. The $C_{release}$ values depended on the initial gellan concentration, C_g, as shown in Figure 3. In the range 1.5% (w/w) $\leq C_g <$ 3.0% (w/w), $C_{release}$ values increased with increasing C_g. In the range $C_g \geq$ 3.0% (w/w), $C_{release}$ values decreased with increasing C_g. The dependence of the release ratio, q, on the initial gellan

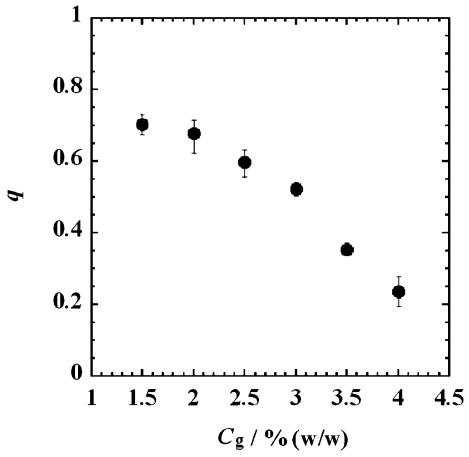

Figure 4. Dependence of the release ratio, q, on the initial concentration of gellan, C_g, in the external solution without added salt at 10 °C. The release ratio, q, is defined as the ratio of the weight of the released gellan chains to the total weight of gellan.

concentration is shown in Figure 4. The q values are defined as follows,

$$q = \frac{C_{release} \cdot W_0}{C_g \cdot W_g} \tag{1}$$

where W_g and W_0 represent the weight of the gel before immersion in the external solution and the weight of the external solution, respectively. The q values monotonically decreased with increasing C_g, suggesting that the amount of released gellan chains decreased with increasing C_g. It is considered that the decrease in the amount of released gellan chains is attributed to the increased counter ion concentration as C_g increases in the gels. As has been shown in previous studies,[9,10] helix formation and the subsequent aggregation of helices forming junction zones are promoted with increasing counter ions. These aggregated helices forming junction zones are linked to the gel network and may not be released out to the external solution. The mobility of molecular chains will be restricted with increasing concentration of gellan, which increased the network density. If the mesh size of the network becomes smaller with increasing concentration of gellan, and if this is the main reason for the decrease in the mobility of unassociated chains, the releasing rate should depend on the gellan concentration, and the amount of the released chains should decrease monotonically as a function of C_g, but this was not the case as shown in Figure 3. In addition to this, only a slight increase in the salt concentration of external solution reduces remarkably the amount of released chain as will be shown in Figure 7. Therefore, the restriction of motion of molecular chains with increasing network density is less important than the counter ion effect which also is increased with increasing concentration of gellan.

Table I. Effect of changing of external solution on release of gellan chain molecules in a 4% (w/w) gellan gel

Number of changes	$C_{release} \times 10^{-2}$, % (w/w)	q^{*a}
1	2.1	0.237
2	0.17	0.256
3	0.02	0.258
4	0.02	0.260

[a] q^* represents total value of release ratio.

The effect of changing the external solution on the release of gellan chains was examined. Table I shows $C_{release}$ values and total values of the release ratio, q^*, for $C_g = 4.0\%$ (w/w) at 10 °C, when the external solution without added salt was replaced every 72 h. The $C_{release}$ values decreased markedly with the change in external solution. At the third and fourth changes, the gellan chains were slightly released from the gels. The q^* value increased slightly at the second change and was almost constant at the third and the fourth changes. The results suggest that in the absence of any temperature increase, unreleased gellan chains fail to be converted into released gellan chains following replacement of the external solution.

The gels from which released gellan chains were removed by replacing of the external solution were dissolved in distilled water by increasing the temperature. The solutions containing unreleased gellan chains were then freeze-dried and a powdered fraction consisting of the unreleased chains was thus obtained. Figure 5 shows the dependence of the release ratio, q, on the initial gellan concentration, C_g, for gels prepared from unreleased gellan chains. As can be seen from Figures 4 and 5, the q value for the unreleased gellan chains was almost equivalent to the value for the mixture of released and the unreleased gellan chains for each initial concentration. The fact that the q values for the unreleased gellan chains were almost equivalent to the values for the mixture for each initial concentration suggests that the counter ion concentration is a dominant factor in determining the amount of gellan chains which can be released from the gels contained in the gels.

Intrinsic Viscosity

In an effort to compare the molecular weight of three fractions of gellan, intrinsic viscosity measurements were carried out in 100 mmol kg^{-1} KCl at 25 °C for the released fraction (released gellan chains), the unreleased fraction (unreleased gellan chains) and the mixture of released and unreleased gellan chains. The released fraction and unreleased fraction were separated by immersing the gel ($C_g = 4.0\%$ (w/w)) in distilled water. The intrinsic viscosities are 0.50 ± 0.07 cm^3 g^{-1}, 1.3 ± 0.1 cm^3 g^{-1} and 1.2 ± 0.1 cm^3 g^{-1} for the released fraction, the unreleased fraction and the mixture, respectively. The intrinsic viscosity of the released fraction is far smaller than that of the other two fractions. It is considered that the molecular weight of the released gellan chains is lower than that of the unreleased gellan chains. The viscosity-averaged molecular weight M_η of a polymer can be evaluated using the Mark-Houwink-Sakurada equation, $[\eta] = KM_\eta^{\alpha}$, if the values of K and α are known for the polymer solution. In the case of sodium-type gellan, the α value was determined to be 1.3 at 25 °C in the 25 mmol kg^{-1} NaCl aqueous solution.[30] The ratio of the molecular weight of unreleased and released gellan chains was evaluated to be *ca.* 3.5 assuming that $\alpha = 1.3$ and that the α value is independent of the type of counter ion and salt concentration added. Ogawa *et al.*[31,32] studied the effect of molecular weight on the conformational change of sodium type gellan molecules, and found that a gellan molecule does not form a helix if the molecular weight is lower than 17,000.

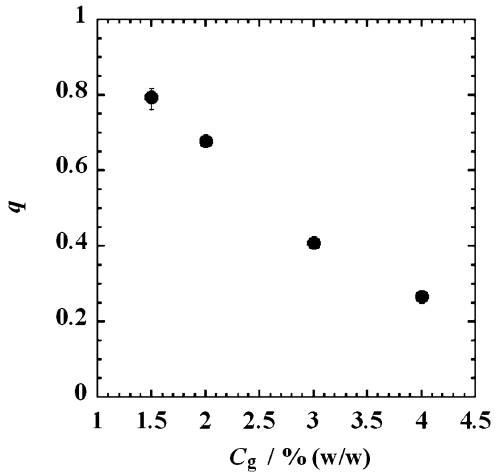

Figure 5. Dependence of the release ratio, q, on the initial gellan concentration, C_g, in the external solution without added salt at 10 °C for the gels prepared from the unreleased gellan chains.

Coil-Helix Transition Temperatures

Cooling DSC curves for solutions of released, unreleased gellan chains and the mixture without added salt at a cooling rate of 0.5 °C/min are shown in Figure 6. For three samples, a single exothermic peak was observed around 30 °C and is attributed to conformational change of gellan molecules from coils to helices. The peak temperatures for the exothermic peak were 29.3 °C, 29.6 °C and 31.2 °C for the mixture, released gellan chains and unreleased gellan chains, respectively. The exothermic peak temperature for the unreleased gellan chains is slightly higher than that of the other samples. The conformation of these gellan chains changed from a coil to a helix at

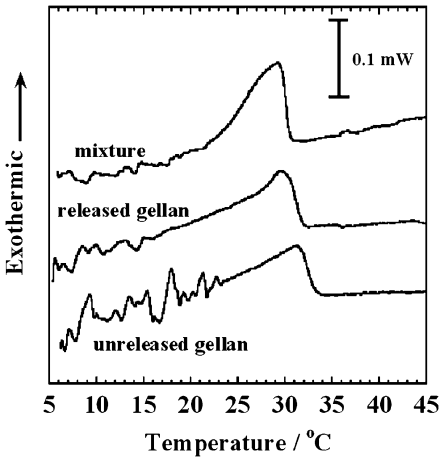

Figure 6. Cooling DSC curves for the solutions of the mixture (upper), released gellan chains (middle) and unreleased gellan chains (lower) at a gellan concentration of 1.0% (w/w) without added salt at a cooling rate of 0.5 °C/min.

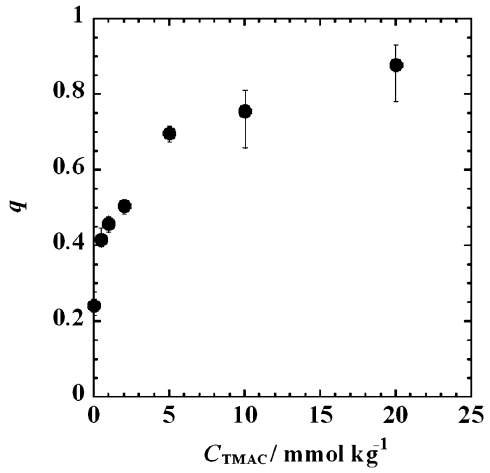

Figure 8. Dependence of the release ratio, q, on the tetramethylammonium chloride concentration, C_{TMAC}, in the external solution for gels with an initial gellan concentration of 4.0% (w/w) at 10 °C.

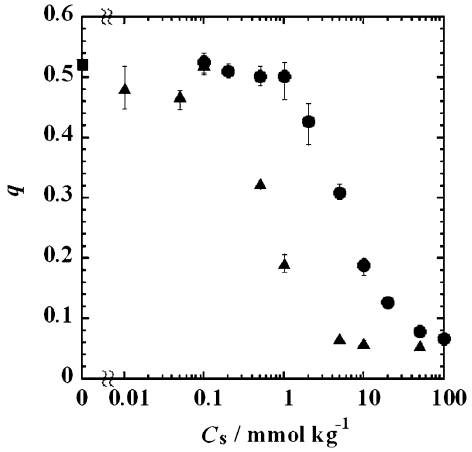

Figure 7. Dependence of the release ratio, q, on the KCl and $CaCl_2$ concentration in the external solution, C_s, for gels with initial gellan concentration of 3.0% (w/w) at 10 °C. (●) KCl. (▲) $CaCl_2$. The square symbol represents the q value in distilled water.

ca. 30 °C on lowering the temperature. Both the released and unreleased gellan chains formed a double helix conformation at 10 °C, at which the q values were measured. The results shown in Figure 6 suggest that the gellan chains which can form a double helix conformation are released from the gels into the external solutions. Although the intrinsic viscosity for the released chains is far lower than that for the mixture, the difference in the intrinsic viscosity is not enough significant to induce the difference in the helix-coil transition temperature as was reported previously.[31,32]

Effect of Added Salts on the Release of Gellan Chains

Figure 7 shows the dependence of the release ratio, q, on the KCl and $CaCl_2$ concentration in the external solution, C_s, for $C_g = 3.0\%$ (w/w) at 10 °C. In the case of the external solutions with added KCl, the q values were almost independent of changes in C_s in the range $0.10\,\text{mmol kg}^{-1} \leq C_s \leq 1.0\,\text{mmol kg}^{-1}$, and they were almost equal to the value obtained in the case of distilled water (square symbol). A marked decrease in the q values was observed in the range $1.0\,\text{mmol kg}^{-1} < C_s \leq 100\,\text{mmol kg}^{-1}$. The q values are likely to plateau in the range $C_s > 100\,\text{mmol kg}^{-1}$. In the case of the external solutions with added $CaCl_2$, the q values decreased markedly with increasing $CaCl_2$ concentration in the range $0.1\,\text{mmol kg}^{-1} \leq C_s \leq 5\,\text{mmol kg}^{-1}$. The q values reached a plateau below 0.1 in the range $C_s \geq 10\,\text{mmol kg}^{-1}$. The results suggest that the amount of released gellan chains decreases following addition of gel-promoting cations such as potassium (K^+) and calcium (Ca^{2+}) ions into the external solutions. Diffusion of gel promoting cations into gels makes the network more dense, and more importantly the increase in the concentration of counter ions, thus suppress the release of chain molecules into external solution as was observed in previous studies.[33–35] The result shown in Figure 7 is consistent with the results shown in Figures 4 and 5. Adding KCl, cesium chloride (CsCl) or $CaCl_2$ facilitate lateral association of gellan double helices.[23–25,36,37] The region of lateral association of double helices plays a role of crosslinking domain in gel network structure. Therefore, it is considered that following diffusion of K^+ or Ca^{2+} into the gels, the double helical gellan chains, which might have been released out in the absence of K^+ or Ca^{2+}, are associated with the crosslinking domains.[38] As a result, the amount of released gellan chains decreases following the addition of K^+ or Ca^{2+}.

Figure 8 shows the dependence of the release ratio,

Table II. Values of E', $C_{unrelease}$ and q of the gellan gels before and after immersion into 100 mmol kg^{-1} KCl at 25 °C and $C_g = 2\%$ (w/w)

	Before immersion	After 74 h immersion
$E' \times 10^5$, Pa	0.11[c]	1.3
$C_{unrelease}$,[a] % (w/w)	0.48	1.9
q[b]	0.76	0.065

[a] $C_{unrelease}$ represents the concentration of unreleased gellan chains in the gel. [b] q values before and after immersion were obtained in the external solution of distilled water and 100 mmol kg^{-1} KCl after 74 h immersion at 25 °C, respectively. [c] The gel was stored for 24 h at 25 °C in mould after gelation at 10 °C.

q, on the tetramethylammonium chloride (TMAC) concentration in the external solution, C_{TMAC}, for $C_g = 4.0\%$ (w/w) at 10 °C. The q values increased with increasing C_{TMAC}. This result suggests that the increase in the amount of released gellan chains is induced by diffusion of TMA^+ into the gels. The gels are completely dissolved when $C_{TMAC} = 50$ mmol kg^{-1} at 10 °C. It has been revealed from atomic force microscopy measurements that TMA^+ prevents lateral association of double helicies.[24,25,34] It is considered that a fraction of the unreleased gellan chains is converted into released gellan chains by disruption of the lateral association of double helical gellan chains following diffusion of TMA^+. It is clear from the results shown in Figures 7 and 8 that the double helical gellan chains, which can be released, are unassociated with the crosslinking domains.

Storage Young's Modulus of Gellan Gels

The measurements of storage Young's modulus, E', of a cylindrical gellan gel were carried out in this study. Table II shows the E' values of gellan gels and the concentrations of unreleased gellan chains, $C_{unrelease}$, before immersion and after 74 h immersion into 100 mmol kg^{-1} KCl solution at 25 °C. No change in diameter of the gel was observed with the immersion. The $C_{unrelease}$ values can be evaluated from the q values at 25 °C, $C_{unrelease} = C_g\,(1 - q)$. The $C_{unrelease}$ and E' values increased by immersion of the gel in KCl solution. It is considered that unassociated gellan chains which should have released out if the gel were not immersed in KCl solution are crosslinked to the gel network by the immersion of the gel into KCl solution.[38] The increasing of $C_{unrelease}$ includes two contributions. The first contribution is originated with conformational change of gellan molecules from coils to helices and the subsequent increase in the number of junction zones. Another one is the formation of junction zones by aggregation of double helical gellan chains. However, the ratio of these two contributions is not clear at present. In both cases, the gellan chains which can be released from the gels are unassociated with the crosslinking domains (junction zones) and then are elastically effective in the gels.

CONCLUSION

In the present study, it has been shown that the fraction of unassociated gellan chains in physical gels can be evaluated from the amount of released gellan chains in the external solution at 10 °C. DSC measurements suggest that released gellan chains adopt a double helical conformation at 10 °C. A comparison of the intrinsic viscosities of released and unreleased gellan chains suggests that the molecular weight of the released gellan chains is lower than that of the unreleased gellan chains. The amount of released gellan chains decreased with increasing concentration of KCl or $CaCl_2$ in the external solution. In the external solution with added TMAC, the amount of released gellan chains increased with increasing TMAC concentration. The results show that the gellan chains, which can be released from the gels, were unassociated with the crosslinking domains composed of the associated double helices in the gels. The conclusion is supported from elastic modulus measurements suggesting that the released gellan chains are not crosslinked to the gel network. The amount of unassociated gellan chains in the physical gels can be determined by a simple method proposed in the present work.

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