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Preparation of carboxymethyl-gellan

Keiichi Miyamoto, Kazuhiko Tsuji, Takato Nakamura, Masayuki Tokita & Takashi Komai

Department of Chemistry for Materials, Faculty of Engineering Mie University, Tsu 514, Japan

In order to obtain a water-soluble gellan derivative, carboxymethyl-gellan was prepared. The carboxymethyl side group was introduced by adding the sodium monochloroacetic acid to gellan under alkaline conditions. The degree of substitution was determined by two different methods; potentiometric titration and infrared spectroscopy. A linear relationship was found between results from the two methods. It was found that the degree of substitution can be controlled by changing the reaction conditions. The solubility of gellan in water is found to increase by the carboxymethylation. The carboxymethyl gellan obtained here dissolved in water at concentrations up to 10% (wt/wt) at 25°C. It is was also found that the solution of the carboxymethyl-gellan does not form a gel when it was cooled to 0°C. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Gellan is the deacetylated extracelluler polysaccharide produced by the microorganism *Pseudomonas eoldea.* Gellan has a tetrasaccharide repeating unit consisting of two β-D-glucose, one β-D-glucronic acid, and α-L-rhamnose linked as shown in Fig. 1. Gellan is a typical anionic heteropolysaccharide since it has a carboxylic group in the repeating unit. This structure has been determined by O'Neill *et al.* (1983). Gellan is known to form a hydrogel under certain conditions. The hydrogel of the gellan shows the thermoreversible sol–gel transition that is similar to other polysaccharides such as carrageenan and agarose (Moorhouse *et al.*, 1981; Kang *et al.*, 1982; Kang & Veeder, 1982, 1983).

Recently, gellan has been used in many areas of food technology. The gel forming ability of the gellan is central to these applications. Hence, to know the mechanism of gelation and how we controlled it is the key to the establishment of these applications. It has been reported that the gelation of gellan occurs when the monovalent and the divalent cation coexist in solution. Because gellan is an anionic heteropolysaccharide, the interaction between the carboxylic group and cations plays an essential role in the sol–gel transition of gellan (Kang & Veeder, 1982 Grasdalen & Smidsrod, 1987). It has already been shown that the concentration of the salts in the gellan solution significantly affects aspects of the gelation of the gellan solution. When the carboxylic group on the gellan is entirely neutralized by gel forming ions, for instance potassium, the solubility of gellan in water drastically decreases. The lower solubility of native gellan is, therefore, assumed to be due to the presence of gel forming ions in the sample. On the other hand, the solubility of gellan in water increases on exchanging gel forming cations with non-gelling cations, such as sodium and tetramethylammonium (TMA) (Grasdalen & Smidsrod, 1987). By using these samples, mechanism of gelation of gellan has been discussed by many researchers. In contrast, no attempt to control the gelation of gellan has been made. A first candidate for this purpose is the chemical modification of the gellan (Crescenzi & Della Valle, 1991; Crescenzi *et al.*, 1992). In this paper, therefore, we would report preliminary results on the preparation of carboxymethyl-gellan (CM-gellan) and its characterization. It is well established in cellulose chemistry that cellulose becomes soluble in water as the degree of carboxymethylation

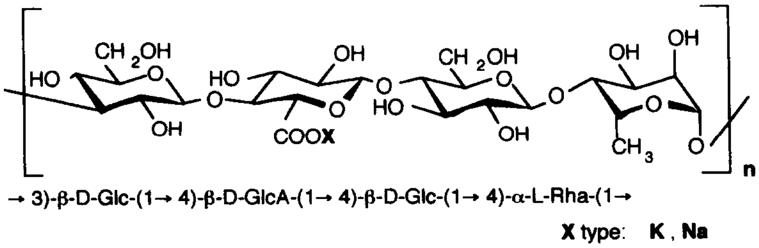

Fig. 1. The idealised repeat unit of gellan.

increases. This phenomenon occurs as a result of the combination of two effects. One is the increase in charge on the cellulose chain, and the other is the destruction of the hydrogen bonds between cellulose chains. We, therefore, expect that these effects also occur when gellan is carboxymethylated. As a result of the carboxymethylation of the gellan, the solubility in water is expected to increase, so that the gelation of gellan can be controlled.

EXPERIMENTAL

Sample preparation

Gellan (potassium salt form) was supplied by San-ei-gen Co. Ltd. The sample was passed through sieves of known sizes. The sample used in this study was in the size range of 106–214μm.

Gellan was dissolved into a sodium hydroxide solution (40wt%) at a concentration of 5wt% (total volume was 20ml). The solution was stirred for 5h at room temperature under vacuo. Crushed ice (50g), made from frozen distilled water, was added to dilute the mixture. The alkaline gellan solution thus obtained was extensively dialyzed against distilled water for 3 days, and freeze-dried to obtain the sodium salt of gellan (Na-gellan).

The CM-gellan was obtained as follows. The alkaline gellan solution was prepared in the same manner as described above. Then the solution of sodium monochroloacetic acid (4M) was successively added to the solution. The temperature of the solution was kept at 0°C. The reaction solution was stirred for 5h, keeping the temperature of the solution at 0°C, and then continuously stirred overnight at room temperature. The solution was extensively dialyzed against distilled water for 3 days, and freeze-dried to obtain the CM-gellan sodium salt.

Characterization

Infrared spectroscopy
The infrared spectra (IR spectra) of these samples were taken with a HITACHI Model 270-50 recording spectrophotometer. The measurements were carried out using KBr method.

Potentiometric titration
The potentiometric titration curves were taken by the reverse titration method using a potentiometer (HORIBA, type H-6 potentiometer). The solution for potentiometric titration was prepared by mixing the following solutions; gellan solution (3ml) adjusted to 1wt% with 0.1N sodium hydroxide solution, 0.1N sodium chloride solution (20ml), and 0.1 N hydrogen chloride (6.5ml). This solution was titrated with 0.01N sodium hydroxide.

Atomic adsorption spectrophotometry
The cation (Na^+, K^+, Ca^{2+} and Mg^{2+}) content of Na- and CM-gellan were analyzed using a Model AA-855 atomic absorption spectrophotometer (Japan-Jyarel-Ash). The adsorption was detected at wavelengths of 589nm (Na^+), 766.5nm (K^+), 422.7nm (Ca^{2+}), and 285.2nm (Mg^{2+}), respectively. Standard solutions (sodium chloride, potassium chloride, calcium chloride and magnesium chloride) were used to calibrate the apparatus. The sample (0.05g) was put into a platinum crucible of 10ml capacity, and then was burned in an electric furnace. The ash obtained was dissolved into hydrogen chloride solution (1N), and then analyzed.

Gel permeation chromatography
The average molecular weights of Na- and CM-gellan were determined by liquid chromatography (FPLC system, Pharmacia). The C-column (diameter = 26mm, length = 700mm, Pharmacia; Bio-gel P-100 (Bio-rad)) was used for this purpose. The sample of 500μl, at a concentration of 5mg/ml, was eluted by de-ionized water at a flow rate of 15ml/h. The elution curves were obtained using a Shodex RI-72 differential refractometer (Showa denkou). Molecular weights of gellan samples were determined by comparing the elution curves of gellan with that of the standard markers; immunoglobulin G (human, m.w. = 1.8×10^5, Wako), serum albumin (bovine, m.w. = 6.0×10^5, Wako), and vitamin B-12 (m.w. = 1.3×10^3, Wako).

RESULTS AND DISCUSSION

Degree of carboxymethylation

The degree of substitution of the carboxymethyl group in the CM-gellan can be determined from both the IR spectra and the potentiometric titration. The values obtained by the IR spectra are, by the nature of the measurements, relative values of the degree of substitution. On the other hand, the values obtained by potentiometric titration correspond to the absolute values of the degree of substitution. It is preferable to determine the absolute values of the degree of substitution sample by sample. However, it is useful to obtain the relationship between the absolute values (potentiometric) and the relative (IR) values from the practical point of view.

The absorption at 1640cm^{-1} is assigned to the stretching vibration of the carboxyl group (C=O) and at 2920cm^{-1} is assigned to the stretching vibration of methin (C-H), respectively. By taking the ratio of these absorption spectra $R_{rel} = A_{1640}/A_{2920}$, we can estimate the relative amount of carboxyl groups in the samples. The value of R is determined from the IR spectra of Na- and CM-gellans that are prepared under various experimental conditions. The results obtained are shown in Table 1. The degree of substitution of the

Table 1. Characterization of carboxymethyl-gellan

	Mol of $ClCH_2COONa$ (/mol of repeating unit)	*Number of carboxyl group (/mol of repeating unit)	Relative I.R. absorbance ($A1640cm^{-1}/A2920cm^{-1}$)	**D.S.
gellan	—	0.715	1.00	0.00
CM-gellan -1	2.7	1.186	1.13	0.07
CM-gellan -2	5.3	2.027	1.27	0.18
CM-gellan -3	13.2	2.099	1.29	0.19
CM-gellan -4	26.5	2.218	1.36	0.20

* Number of carboxyl group determined by potentiometric titration.
** Degree of etherealization.

CM-gellan, $D.S._{rel}$, is calculated by the following equation.

$$D.S._{rel} = R_{rel} - 1$$

Here, the numerical constant is chosen as the value of $D.S._{rel}$ becomes zero for the original gellan sample.

The absolute values of the degree of substitution of these samples are also determined by the potentiometric titration. The total number of carboxyl group is determined from the equivalence point on the titration curve of each sample. The number of carboxyl groups in the repeating unit of gellan is one, assuming an ideal repeating unit. The maximum number of the carboxyl group on the CM-gellan is ten, since there are ten possible sites of substitution (–OH group) on an ideal repeating unit of gellan. The absolute value of the degree of substitution, $D.S._{abs}$, is, therefore, calculated by the following equation.

$$D.S._{abs} = 0.1 \times (total[COOH]\ of CM-gellan) / (total[COOH]\ of gellan)$$

Here, the numerical factor, 0.1, is chosen so that the value of the degree of substitution becomes unity when all hydroxyl groups on the repeating unit of gellan are substituted by the carboxyl groups.

The absolute values of the degree of substitution obtained are plotted as a function of the relative value of the degree of substitution in Fig. 2. It is clear from this figure that the absolute value of the degree substitution is proportional to the relative value. The least squares analysis of the results, shown in Fig. 2, yields the following relationship.

$$D.S._{abs} = 0.62 \times D.S._{rel}$$

The absolute value of the degree of substitution is, therefore, determined from the IR spectrum of the CM-gellan by the above equation.

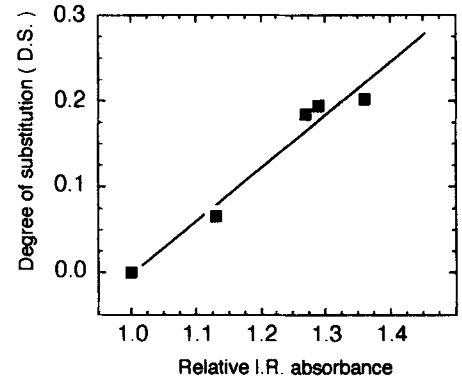

Fig. 2. The relationship between the degree of substitution determined by potentiometric titration and IR spectroscopy.

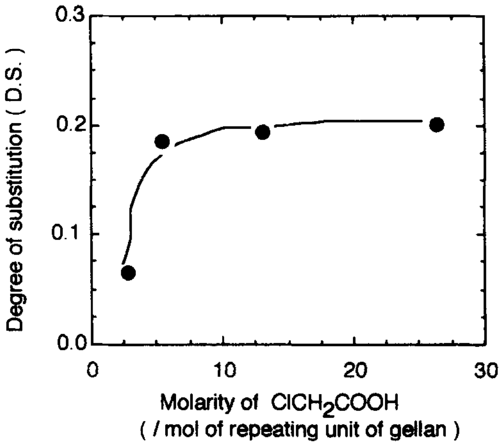

Fig. 3. The relationship between the degree of substitution of the CM-gellan and the reaction conditions. The horizontal axis indicates the molarity of monochloroacetic acid (mol/mol of repeating unit of gellan) added to the reaction system. The carboxymethylation is made under the same experimental conditions except for the amount of the monochloroacetic acid.

It may be important to control the degree of substitution of the carboxmethyl group on the repeating unit of gellan, for the practical purpose of controlling the physical and chemical properties of the solution as well as the gel. In the case of carboxymethylation, this purpose is easily achieved. It is found that the degree of substitution changes with the composition of the reactant. Figure 3 shows the relationship between the degree of substitution and the molarity of the sodium monochloroacetic acid added to the reaction system. It is clear from this figure that the degree of substitution increases on increasing the amounts of sodium monochloroacetic acid in the reaction system. The asymptotic value of the degree of substitution is found to be approximately 20% above 3 mol of sodium monochloroacetic acid.

Ions in the CM-gellan

The number of the cations that are contained in the Na- and the CM-gellan prepared here is shown in the Table

Table 2. Metal ion content of gellan (original sample K form), Na-gellan and CM-gellan. [Unit: mol of ions/mol of repeating unit of gellan]

	Na	K	Ca	Mg
gellan (K^+ salts)	1.22	0.76	1.7×10^{-2}	5.1×10^{-3}
gellan (Na^+ salts)	0.72	5.8×10^{-3}	9.3×10^{-3}	3.3×10^{-2}
CM-gellan (Na^+ salts)	1.31	3.5×10^{-3}	5.6×10^{-3}	1.9×10^{-2}

2. The amount of sodium ion that is contained in the Na-gellan is 0.72 for a repeating unit of gellan. On the other hand, the absolute amount of the carboxylic acid, which is determined by the potentiometric titration, is found to be 0.72. These results indicate that all the carboxyl groups in the gellan are entirely replaced by the sodium ions. The other cations contained in the sample gellan are completely removed by dialysis in the Na-gellan prepared here. In the case of the CM-gellan, similar results are obtained. These results suggest that the Na- and CM-gellan obtained here are, to a first approximation, of the sodium salt form.

Molecular weight of carboxymethyl-gellan

The molecular weights of Na- and CM-gellan prepared here are shown in Table 3. The molecular weight of Na-gellan is found to be 1.7×10^5. In the case of the CM-gellan ($D.S._{abs}$ 0.19), the elution pattern shows two maxima,corresponding to the molecular weight of 0.6×10^5 and 1.3×10^5, respectively. These results suggest that the degradation of the gellan chain occurs during the chemical modification process, since the molecular weight of the CM-gellan is smaller than that of Na-gellan.

Solubility and gelation properties

The solubility of the gellans was studied. The maximum concentration of the aqueous solution of the original gellan (potassium form), which we can prepare, is less than a few percent in weight. In addition, a temperature of 90°C is necessary to stabilize this material. In contrast, the Na- and CM-gellan are found to be soluble in water at room temperatures. The maximum concentration of solutions of Na- and CM-gellan that can be prepared at at a temperature of 25°C was found to be 4.0 and 10wt%, respectively. The increased solubility of the CM-gellan results from a combination of molecular weight reaction, ion exchange and chemical modification. It is clear that the exchange of the potassium ion by the sodium ion enhances the solubility of the gellan. The charge density on the polymer chain is also increased by the carboxymethyl group in the case of CM-gellan.

Table 3. Molecular weights of gellan and CM-gellan determined by gel permeation chromatography. (Table shows molecular weight at elution pattern maximum)

	Molecular weight ($\times10^5$)
gellan (Na salts)	1.7
CM-gellan (D.S. 0.2*)	1.3
	0.6

* The elution pattern of the CM-gellan shows two main peaks.

It is also found that the Na-gellan solution transforms into gel at a temperature of 0°C, without adding any salts. On the other hand, the CM-gellan solution does not form a gel up to 10wt.% even when the temperature is lowered to 0°C. Furthermore, it is found that only the addition of potassium ions promotes the gelation of CM-gellan solutions. The stability of the gellan gel has been extensively studied. The results gained so far strongly suggest a selective interaction between gellan and potassium ions. The difference in the gel forming ability of cations is slight in the case of native gellan. However, the difference in the gel forming ability of gellan is enhanced by the substitution of carboxylmethyl group into native gellan. Therefore, this may suggest selective interaction between the gellan and the potassium ions. Further detailed studies of the gelation properties of the CM-gellan will promote a better understanding on the gelation mechanism of the native gellan. Such studies are now in progress.

CONCLUSION

The sodium salt of carboxymethyl gellan is prepared by a simple reaction method. The degree of the carboxymethyl substitution is determined by two different techniques. The results obtained by both methods are in good agreement with each other. It is found that the solubility of the CM-gellan obtained is much higher than that of gellan. The gel forming ability of the CM-gellan is different from that of the native gellan, i.e. only the potassium ions promote the gelation of the CM-gellan. This suggests a selective interaction between gellan and potassium ion.

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