Salt-induced extension and dissociation of a native double-stranded xanthan

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(Received 9 December 1985)

This study shows that xanthan molecules at room temperature may assume at least three different conformations in 0.1 M NaCl aqueous solutions in which the local structure is ordered: (1) the native compact double helix, (2) the extended double helix, and (3) the extended single helix. Experiments including viscosity, low-angle light scattering and optical rotation measurements have been carried out with a fully pyruvated and fully acetylated native laboratory sample supplied as fermentation broth. Two major conformation changes of the native double helix which were found irreversible in our experimental conditions can be induced by treatments at low ionic strength. After treatment in 10^{-4} M NaCl, xanthan is still a double helix in 10^{-1} M NaCl, but the backbone of each strand has been extended. After the sample has been in 10^{-5} M NaCl, the double helix has been dissociated and a single helix sample is obtained. Thus, the denaturing of xanthan is a two-step process. The first step consists of the extension of the two chains inside the double helix, and the second is a dissociation of the native double strand.

Keywords: Polysaccharides; xanthan; double-stranded; salt-induced extension

Introduction

The primary structure of xanthan has been clearly established and consists of a linear (1→4) β-linked D-glucan chain substituted on every glucose residue by a trisaccharide side chain[1,2]. In the native form every internal mannose has a variable degree of pyruvate substitution which is thought to be dependent on both the bacterial strain and fermentation conditions[3,4].

The secondary structure, which can be more or less ordered, has been shown to depend on physicochemical conditions (pH, salinity and temperature) and on the primary structure (i.e. acetate and pyruvate content)[5,6]. Most authors explain the stabilization of the more ordered conformation by a packing of the side chains along the backbone by H-bonding.

The question of whether the xanthan molecule is a single or a double strand in an aqueous solution is still being debated. Among the data reported in the literature in recent years, an X-ray study[7] and an analysis of the thermally induced order–disorder transition[8-10] supports the hypothesis of a single strand. Other results based on the characterization of xanthan fractions prepared by sonication[11-13] suggest a double-stranded structure. In 1984 convincing, and apparently contradictory, papers were published[14-16]. In the first one, we claimed that a fully pyruvated laboratory sample manufactured and pasteurized by Rhône-Poulenc was a single helix with a persistence length of 50 nm and a mass-per-unit length of 10^3 dalton/nm when dissolved in aqueous salt solution (0.1 M NaCl, $T=30$°C, i.e. in an ordered form). This statement was established from an analysis of the relationship between intrinsic viscosity and molecular weight, based on the wormlike chain model. The experiments were carried out on fractions obtained by a new technique of surface exclusion chromatography[17]. On the other hand, Sato *et al.* published two successive papers[15-16] showing that:

(1) a sample manufactured by Kelco Co. was a double helix in an aqueous solution having the same salinity (0.1 M NaCl) with a persistence length of 120 nm and a mass-per-unit length of 1.94×10^3 dalton/nm;

(2) this double strand could be separated into single chains either by dissolution in cadoxen or by being heated to 95°C in distilled water.

The present paper deals with the conformational changes of a fully acetylated and pyruvated native xanthan sample induced by treatments at low ionic strength at room temperature. The variations with salinity of molecular weight, intrinsic viscosity and optical rotation are analysed and interpreted. Two main conformational changes are described. The first consists of an extension of the native double helix after the molecule has been dissolved with a salt concentration low enough to get a disordered structure. The second is a dissociation of the native double helix which can be obtained after dissolution at very low ionic strength. These conformation changes were found irreversible in our experimental conditions. The results of this study may explain apparent disagreements among different authors concerning the question of whether the xanthan is dissolved in aqueous solution as a single or a double strand. Indeed, it is shown that these apparent disagreements can be due to the differences in the treatments the samples underwent before the study.

0141–8130/86/050306–05$03.00

Experimental

Experimental techniques

Polymer concentrations were accurately determined with a Dohrmann DC80 carbon analyser. Molecular weights were measured at low angle (5°) with a Chromatix KMX6 photometer. Refractive index increments were determined at 633 nm with a Chromatix KMX16 differential refractometer at constant chemical potential obtained either by dialysis or by ultrafiltration technique.

Preparation of polymer solutions

The xanthan sample used was manufactured by Rhône-Poulenc, supplied as a fermentation broth without pasteurization, and thus is a truly native product.

A stock solution at $C = 400$ mg/l was prepared by simple dilution of the broth with 0.1 M NaCl and was prevented against bacterial degradation by adding 400 mg/l of sodium azide. This solution was clarified by successive high-differential pressure (1 bar) filtrations through Millipore filters (3 and 0.8 μm) to remove bacteria cells. The filterability of the solutions thus prepared was found to be excellent as checked by using a standard test previously described[18]. Nevertheless, any possible remaining microgels in these translucid solutions were eliminated by using a very low shear rate filtration method which has previously been described in detail[18]. Most of the proteins were removed by absorption on Millipore filters during this filtration treatment since titrations using the Bradford method[19] showed that the protein–polymer ratio in purified solutions is lower than 1%. Subsequent ultrafiltration using weakly adsorbing Ultrapore membranes (Type C) was performed either to eliminate very low molecular weight impurities (i.e. $\bar{M}_w < 20\,000$) or to adjust the salinity to the desired level. The characteristics of the xanthan sample and of its aqueous solutions under conditions where an ordered conformation prevails (0.1 M NaCl, pH = 7, $T = 30$°C) are given in *Table 1*.

Treatments at different salinities

After removing bacteria cells and microgels as described above, an ultrafiltration technique was used to prepare xanthan solutions at $C = 200$ mg/l having salinities varying between 10^{-5} and 1 M NaCl. For each solvent salt concentration, the polymer solution was ultrafiltrated until the conductivity of the solvent was found to be the same at the inlet and at the outlet of the ultrafiltration cell. After this step, NaCl was added or removed to give 0.1 M NaCl salinity for measuring molecular weight, optical rotation and intrinsic viscosity.

Results and discussion

Molecular weight

Average molecular weights of three aqueous solutions A, B, and C were determined by light scattering. They were of the same salinity (0.1 M NaCl) but had been subjected to treatments consisting of steps of dissolution at different ionic strengths but at 200 mg/l polymer concentration (*Figure 1*). These molecular weight measurements were repeated several times for distinct preparations of A, B and C solutions. The native xanthan (curve A) which was maintained at salinities equal to or higher than 0.1 M NaCl has a molecular weight of $4.8 \pm 0.1 \times 10^6$. When xanthan was treated in 10^{-3} or 10^{-4} M NaCl and then its salinity readjusted to 0.1 M NaCl (curve B), a slightly lower molecular weight, $\bar{M}_w \approx 4.1 \pm 0.1 \times 10^6$, was found. This variation in molecular weight suggests the dissociation by electrostatic repulsions of some aggregates possibly existing in the native sample solutions. Finally when xanthan was dissolved in 10^{-5} M NaCl, i.e. practically in pure water (curve C), the molecular weight measured at 10^{-1} M NaCl was roughly halved, $M_w \approx 2.1 \pm 0.1 \times 10^6$. The conclusion was thus reached that the double-strand native xanthan can be dissociated by extensive salt removal. The fact that such complete dissociation could be achieved without heating is due to the high pyruvate content of our sample. In 10^{-5} M NaCl solutions, the two strands of the native xanthan were separated by long distance interchain electrostatic repulsions between the charged groups in these two strands. Since the scattered light was found not to increase with time even after 24 h, at $C = 200$ mg/l, for the three solutions A, B and C we conclude that no time-induced aggregation occurs for this pure and highly pyruvated sample, contrary to previous observations[9,20]. Moreover, a monostranded sample could be conserved at low temperature for several years without any increase in molecular weight, showing that dissociation is completely irreversible[24].

The dissociation of the native double strand is in good agreement with the results of Sato, who found such dissociation of the xanthan molecule both in pure water at 95°C and in cadoxen[16]. Since the sample used by Sato had a low pyruvate content ($DS_{pyr} \approx 0.3$), the electrostatic

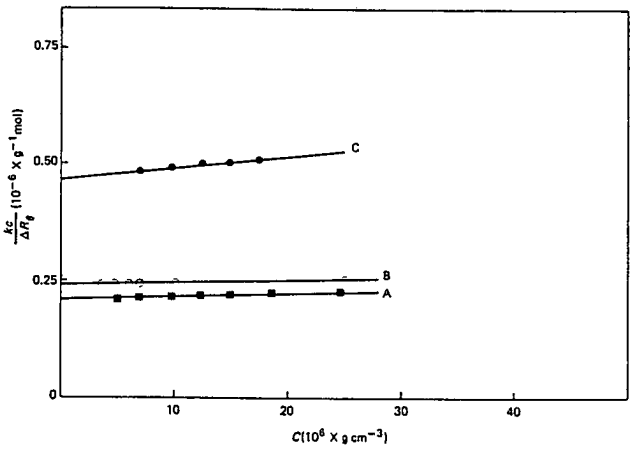

Figure 1 Low angle light scattering behaviour of the native xanthan in 0.1 M NaCl without low salinity treatment (A), after treatment at 10^{-4} M NaCl (B), and at 10^{-5} M NaCl (C)

Table 1 Native xanthan solution characteristics in 0.1 M NaCl

$\bar{M}_w$	DS Pyruvic	DS Acetyl	A_2 (g^{-2}mol cm^{-3})	dn/dc (ml/g)	$[\eta] \rightarrow 0$ (cm^3/g)
4.8×10^6	0.98	0.99	4×10^{-4}	0.15_5	3400

repulsions between the two strands were weaker than for our fully pyruvated sample and thus, the energy required for dissociation of the double helix was obtained in part by an increase in temperature. In the same way, it is likely that the native xanthan sample studied by Milas and Rinaudo[21] was actually a double helix. The absence of molecular weight variation observed in the salinity and temperature range of their experiments can be explained by the too low pyruvate content of their sample ($DS_{pyr} \approx 0.4$). Moreover, the highly pyruvated sample which we previously characterized as a single helix[14] had been pasteurized and thus had been dissociated into two single strands during this treatment.

This analysis, which points out that the xanthan molecule can be a double or a single strand depending on the temperature and the salinity of the aqueous solutions at which the native polymer has previously been treated, suggests an explanation for apparent discrepancies occurring in the literature concerning the structure of xanthan. For example, the sample manufactured by Kelco Co. ($DS_{pyr} = 0.5$) and shown by Norton to be a single helix[9] had been heated to 80°C in pure water before its structure was characterized. This treating step probably dissociated the native double strand. On the other hand, in the experiments by Brant[13] which were performed with a similar Kelco product dissolved in water but at room temperature or in those by Holzwarth[12] performed with a sample purified only by precipitation in acetone, the double-stranded native structure was preserved.

Optical rotation

The variations of the specific optical rotation $[\alpha]$ are plotted *versus* salinity in *Figure 2*. In a first series of experiments, where the polymer solution salinity was decreased from 1 to 10^{-4} M NaCl (curve A) and then increased to 1 M NaCl (curve B), i.e. under conditions where the double-stranded structure is preserved as checked by molecular weight determination, the absolute value of $[\alpha]$ decreased from 35° at 0.1 M NaCl down to a value near 20° at 10^{-4} M NaCl and then increased to a value close to 50° by coming back to 0.1 and 1 M NaCl. This irreversible change in optical rotation implies an irreversible structural change of the native double strand we interpret as a reorganization of H-bonding between the trisaccharide side chains and the backbone, giving a higher order after treatment at low salinity than in the native form. It must be noted that after this first denaturation, the salt-induced variations in optical rotation were found to be reversible (curve B) if the salinity remains higher than 10^{-4} M NaCl.

In a second series of experiments the salinity was reduced down to very low values (10^{-5} M NaCl), and a separation between the two native strands was achieved as shown by halving the molecular weight (see above discussion and *Figure 1*). The variations of $[\alpha]$ with salinity (curve C) are entirely reversible and are similar to those observed with the single-stranded sample we studied previously[24]. Light scattering measurements have checked that these $[\alpha]$ variations occur without any change in molecular weight, both in this study and in Ref. 24. The order–disorder transition of this fully acetylated xanthan occurs at a lower salinity (10^{-3} M NaCl) than that of the pasteurized sample studied previously (10^{-2} M NaCl)[24], the acetylation degree of which was much too low ($D_{ac} \simeq 0.1$) due to the pasteurization process. This behaviour is consistent with literature observations showing that acetyl groups stabilize the ordered form[6]. Moreover, the transition (*Figure 2*, curve C) is smoother than that obtained with the less acetylated xanthan[24]. Indeed, the enthalpy variation associated with the transition increases with the acetate content of the xanthan molecule, what can explain, by applying the model proposed by Norton[10], an increase in the salinity course of conformational ordering for a more acetylated sample.

The irreversible increase in optical rotation we observed after the native double helix has been in disordered conformation was not found by Milas and Rinaudo[21] who studied a sample having a lower pyruvate content. This difference suggests that the organization of H-bonding between the side chains and the backbone is strongly dependent on the presence of pyruvic groups which increase both electrostatic repulsions and steric hindrances.

Intrinsic viscosity

The variations in the zero shear rate intrinsic viscosity, $[\eta]_0$, measured at 0.1 M NaCl salinity as a function of the minimum salinity $[NaCl]_m$ of the solution in which xanthan was treated are shown in *Figure 3*. A strong and irreversible increase in $[\eta]_0$ from 3400 to 8500 $cm^3\ g^{-1}$ is

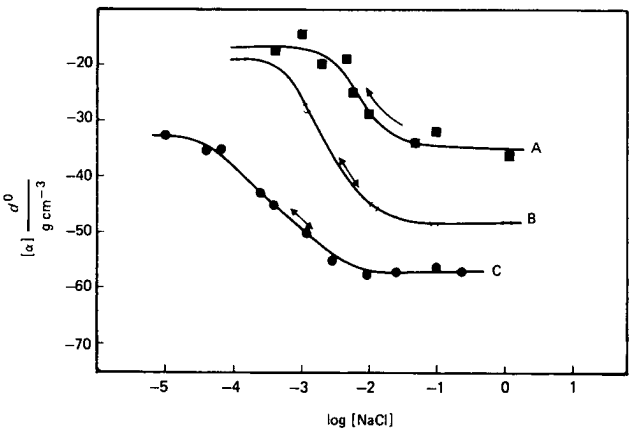

Figure 2 Salinity dependence of specific optical rotation for native xanthan (A), xanthan treated at 10^{-4} M NaCl (B) and at 10^{-5} M NaCl (C)

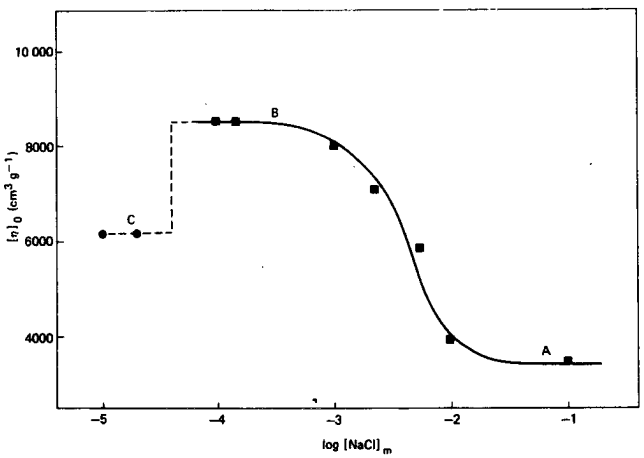

Figure 3 Intrinsic viscosity at zero shear rate of the native xanthan sample in 0.1 M NaCl as a function of the salinity treatment $NaCl_m$ given in mol/l. (■ double strand, ● single strand)

observed after the xanthan has been treated in solutions with low salinity (10^{-3} and 10^{-4} M NaCl) where the disordered conformation prevails (see *Figure 2*). The irreversibility of this viscosity increase in 0.1 M NaCl solution has been checked over a period of three months. Such an increase in viscosity similar to that observed by Milas and Rinaudo[21] suggests that the structural change displayed by the increase in optical rotation is an extension of the native compact double helix. Comparable structural changes between a compact and a more extended conformation of a single strand have been predicted to occur from conformational calculations[22]. The high value of $[\eta]_0$ (8500 $cm^3 g^{-1}$) may be attributed to the extended conformation (about 9 Å per disaccharide residue), which is also close to the one which has been measured by X-ray diffraction[7]. By using Yamakawa calculations[25], we found the persistence length of our sample in the extended double strand conformation equal to 150 nm, a value slightly higher than that previously reported by Sato[16] for a less pyruvated double-stranded xanthan sample.

Recent calculations[22] predict that one of the lowest energy conformations of xanthan is a very compact structure with a projection of one disaccharide residue along the helix axis of about 1.5 Å. From the experimental value $[\eta]_0 = 3400\ cm^3 g^{-1}$ measured for our native sample and by assuming the xanthan molecule is a strictly rigid rod, the actual value of the length per disaccharide residue is higher, about 3 Å. Indeed, it seems very unlikely that the compact conformation theoretically predicted can exist all along the molecule as suggested by Perez[23].

When salinity is decreased down to 10^{-5} M NaCl, the native double strand is dissociated and a strong decrease in $[\eta]_0$ is observed, implying an increase in molecule flexibility. Indeed, the wormlike chain model[24] gives a value of the persistence length equal to 60 nm in 0.1 M NaCl, for a single strand, a value three times shorter than that obtained for the double strand. This value is slightly larger than that previously determined for a fully pyruvated single stranded sample having a lower acetate content (50 nm, see Ref. 24).

Figure 4 shows the variations in the intrinsic viscosity as salinity decreases. Between 10^{-1} and 10^{-3} M NaCl, the electrostatic persistence length is negligible compared with the structural one calculated above ($q = 150$ nm) and thus the $[\eta]_0$ variations are due to the conformational change of the compact helix to a more extended one, without any dissociation of the double strand. The strong increase of $[\eta]_0$ observed between 10^{-3} and 10^{-4} M NaCl can be attributed to the increase in the electrostatic persistence length according to a mechanism previously described for a single-stranded sample (see *Figure 3* and Ref. 24). A tendency to reach a constant value of $[\eta]_0$ around 10^{-4} M NaCl is found, probably because the maximum extension of the xanthan chain has been reached as in the case of a single strand[24]. This maximum value of $[\eta]_0$ is very high suggesting the occurrence of electroviscous effects due to the difference of motion of the polymer with respect to its counterion atmosphere, as observed for other great linear charge density polymers[26].

The value of $[\eta]_0$ ($18 \times 10^3\ cm^3 g^{-1}$) found for this sample ($\bar{M}_w \approx 2.1 \times 10^6$) in 10^{-5} M NaCl when the double helix has been dissociated is quite consistent with the one previously determined for a slightly lower molecular weight sample ($\bar{M}_w \approx 1.8 \times 10^6$, $[\eta]_0 = 15 \times 10^3\ cm^3 g^{-1}$ Ref. 24).

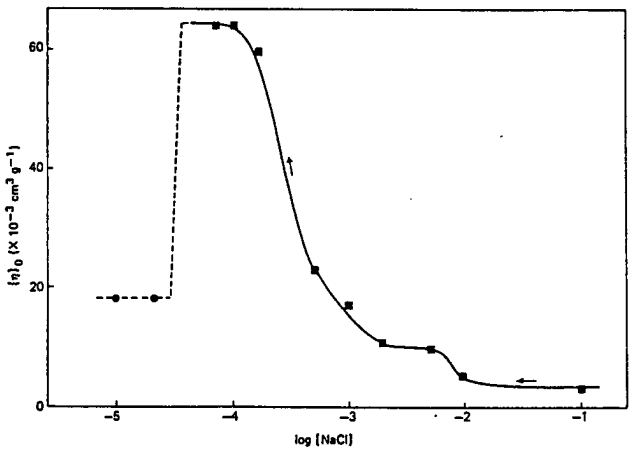

Figure 4 Salinity dependence of intrinsic viscosity at zero shear rate of the native xanthan sample (■ double strand, ● single strand)

Conclusion

This study clearly shows that xanthan may assume at least three different ordered conformations in 0.1 M NaCl aqueous solutions at 30°C: (1) a native compact double helix, (2) an extended double helix, and (3) an extended single helix.

These different conformations were obtained by treatments at different salinities at room temperature for a fully pyruvated and acetylated native xanthan sample. The salt-induced order–disorder transition occurs without any change in molecular weight, even for a double-stranded sample, and thus is an intramolecular process inasmuch that the salinity is not decreased below 10^{-4} M NaCl. If salinity is decreased at lower values, the dissociation of the native double helix into two single chains occurs as a result of the electrostatic repulsions between charged groups located on the two different strands. Whatever the conformation and the salinity, no time-dependent aggregation of xanthan molecules was detected with the sample used.

The above experimental results enabled us to make an analysis of literature data which showed that apparent discrepancies between the xanthan structure studied arose only from differences in salinities and temperatures of the treatments that the samples underwent.

Acknowledgements

The authors thank Mr Cros from Rhône-Poulenc Industries for providing polymer samples and for helpful suggestions. They are indebted to S. Perez for very fruitful discussions.

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