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Alkali-modification of carrageenans: mechanism and kinetics in the kappa/iota-, mu/nu- and lambda-series

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The cyclization reaction (formation of 3,6-anhydro-α-D-galactose units from α-D-galactose 6-sulfate) of carrageenans follows a pseudo first-order kinetics, being 20–60 times faster for carrageenans of the kappa-family than for those of the lambda-family. In lambda-carrageenans the clustering of the sulfate groups around the hydroxyl on C-3 of the α-unit shields it from polarization or ionization, reducing the cyclization reaction rate. Furthermore, molecular models of lambda-carrageenans suggest an adequate geometry for the interaction between the hydroxyl group on C-3 of the α-unit and the sulfate group on C-2 of the β-unit. These observations also explain the lack of cyclized derivatives of lambda-carrageenans in nature. The ease with which the cyclization reaction occurs for carrageenans of the kappa-family indicates that the alkaline treatments used industrially could be carried out under milder conditions, giving products of high gel strength.

INTRODUCTION

The formation of 3,6-anhydro-α-D-galactose units from α-D-galactose 6-sulfate residues by alkaline treatment is an important and well-known reaction undergone by carrageenans (Percival, 1949; Turvey, 1965). It is used commercially to enhance gelation behavior (Glicksman, 1983) and in the laboratory for the quantitative determination of α-galactose 6-sulfate residues (Rees, 1961). In spite of its importance, the mechanism and kinetics of the reaction have not been extensively studied (Percival, 1949; Turvey, 1965). Here we report the rates of cyclization for different types of 'pure' carrageenans obtained from cystocarpic and tetrasporic plants of *Gigartina skottsbergii* (Matulewicz *et al.*, 1989) and correlate these data with their structures.

EXPERIMENTAL

Materials

Cystocarpic and tetrasporic plants of *Gigartina skottsbergii* were collected in Bahía Camarones (Provincia de Chubut, Argentina) and sorted in the Instituto Nacional Patagónico (CONICET; Puerto Madryn, Chubut).

Extraction and fractionation of carrageenans

The extraction, fractionation and analysis of carrageenans have been described elsewhere (Matulewicz *et al.*, 1989).

Alkaline treatment

The general procedure for the analytical treatment was carried out as described by Matulewicz *et al.* (1989). The sample (50 mg) was dissolved in water (25 ml) and sodium borohydride (2·5 mg) was added. After 24 h at room temperature, 3 M sodium hydroxide was added (12·5 ml), together with a further quantity of sodium borohydride (1·5 mg) to give a final concentration of 1 M sodium hydroxide. The resulting solution was divided into 1·0-ml aliquots which were placed in sealed tubes and heated at 80°C. Samples were taken at regular intervals and the reaction was stopped by cooling in an ice bath; the solutions were neutralized with 1 M

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hydrochloric acid and the 3,6-anhydrogalactose content was determined by the resorcinol method (Yaphe, 1960). From these results, the rate constants and half-lives were determined. This experiment was repeated at different temperatures (50–90°C).

The standard deviation was determined for the rate constants and half-lives, and an average of 5–10% was estimated.

This reaction was carried out at other sodium hydroxide concentrations and with addition of sodium sulfate in order to modify the ionic strength.

RESULTS

The alkaline treatment was carried out with kappa/iota-carrageenans ($1C_1$ and $1C_2$), a partially cyclized mu/nu-carrageenan ($1C_3$), and lambda-carrageenans ($1T_1$ and $1T_2$) (Matulewicz *et al.*, 1989, 1990; Ciancia *et al.*, in press). Table 1 shows the composition (molar ratio Gal:3,6-AnGal:sulfate) and the molar percentages of 4-linked galactose 6-sulfate and 2,6-disulfate units present in these carrageenans.

For $1C_1$ and $1C_2$ the reactions were completed after 2 and 4 min, respectively; the small amount of 4-linked 6-sulfated galactose units in both carrageenans precluded a kinetic analysis.

The cyclization reaction follows a pseudo first-order kinetics as determined by the plot of $(\ln A_0 - A_\infty/A_t - A_\infty)$ as a function of time, where A is the absorbance determined by the resorcinol test (Fig. 1). Table 2 shows the rate constants and half-lives of this reaction for $1C_3$, $1T_1$, and $1T_2$ in 1 M sodium hydroxide at different temperatures.

The cyclization reaction is about 20–60 times faster for carrageenans of the kappa-family ($1C_3$) than for lambda-carrageenans ($1T_1$ and $1T_2$). Differences in the rate constants for $1T_1$ amd $1T_2$ are possibly related to the higher sulfate content of the last fraction.

The influence of the concentration of alkali and the ionic strength are given in Tables 3 and 4, respectively. From the plot of the pseudo first-order rate constant as a function of the sodium hydroxide concentration the real second-order rate constant of the reaction was determined for $1C_3$ ($k_2 = 5{\cdot}2 \times 10^{-4}$ liters mol^{-1} s^{-1}). The second-order rate constant for $1T_2$ increases from $0{\cdot}14 \times 10^{-4}$ liters mol^{-1} s^{-1} in 1 M sodium hydroxide to $0{\cdot}37 \times 10^{-4}$ liters mol^{-1} s^{-1} in 4 M sodium hydroxide, and then remains constant. A higher pseudo first-order rate constant is obtained when the ionic strength of the medium is increased with sodium sulfate, but this increment is lower than that observed on raising the ionic strength to the same value with alkali alone. It is noteworthy that the increments are higher for $1C_3$ than for $1T_2$.

DISCUSSION

Carrageenans of the kappa-family react considerably faster than those of the lambda-family (Table 2): the real second-order reaction rate is about 40-times higher for the former in 1 M sodium hydroxide and 15-times higher at 4–6 M alkali concentration. Besides, higher ionic strengths also increase the reaction rates (Tables 3 and 4).

The cyclization reaction of the α-D-galactose 2,6-disulfate units to the corresponding 3,6-anhydro derivatives in a carrageenan molecule involves several consecutive steps: (1) the polarization or ionization of the hydroxyl group on C-3, producing the repulsion of the equatorial oxygen on C-3 and sulfate on C-2, and, consequently, the destabilization of the $^4C_1$ conformation of these units; (2) the change to the $^1C_4$ conformation in an attempt to place the groups on C-2 and C-3 as far away as possible; in this arrangement the oxygen on C-3 and the sulfate group on C-6 are in axial parallel positions; (3) the intramolecular S_N2 reaction with the formation of the 3,6-anhydro ring and the concomitant displacement of the C-6 sulfate group; and finally (4) the re-accommodation of the conformation of the whole molecule. The driving force of this reaction is, thus, the polarization or ionization of the hydroxyl group on C-3 of the α-D-galactose 2,6-disulfate unit, in agreement with the experimental data.

Carrageenans of the kappa- and lambda-families differ in the amount and distribution of the sulfate groups (Painter, 1983); thus, in lambda-carrageenans

Table 1. Analyses of carrageenans $1C_1$, $1C_2$, $1C_3$, $1T_1$, and $1T_2$

Sample[a]	Carrageenan	Gal:3,6-AnGal:sulfate molar ratio	Sulfate (mol per 100 units)	α-Gal-6-sulfate[b] (mol per 100 units)	α-Gal-2,6-disulfate[b] (mol per 100 units)
$1C_1$	kappa/iota	1·00:0·64:1·32	80·5	—	4·3
$1C_2$	kappa/iota	1·00:0·62:1·20	74·1	3·1	5·6
$1C_3$	mu/nu	1·00:0·37:1·14	82·0	7·3	21·2
$1T_1$	lambda	1·00:0·03:1·12	108·7	—	44·5
$1T_2$	lambda	1·00:0·64:1·20	116·5	—	61·9

[a] C-Fractions and T-fractions were isolated from cystocarpic and tetrasporic plants of *Gigartina skottsbergii*, respectively.
[b] Determined according to Rees (1961).

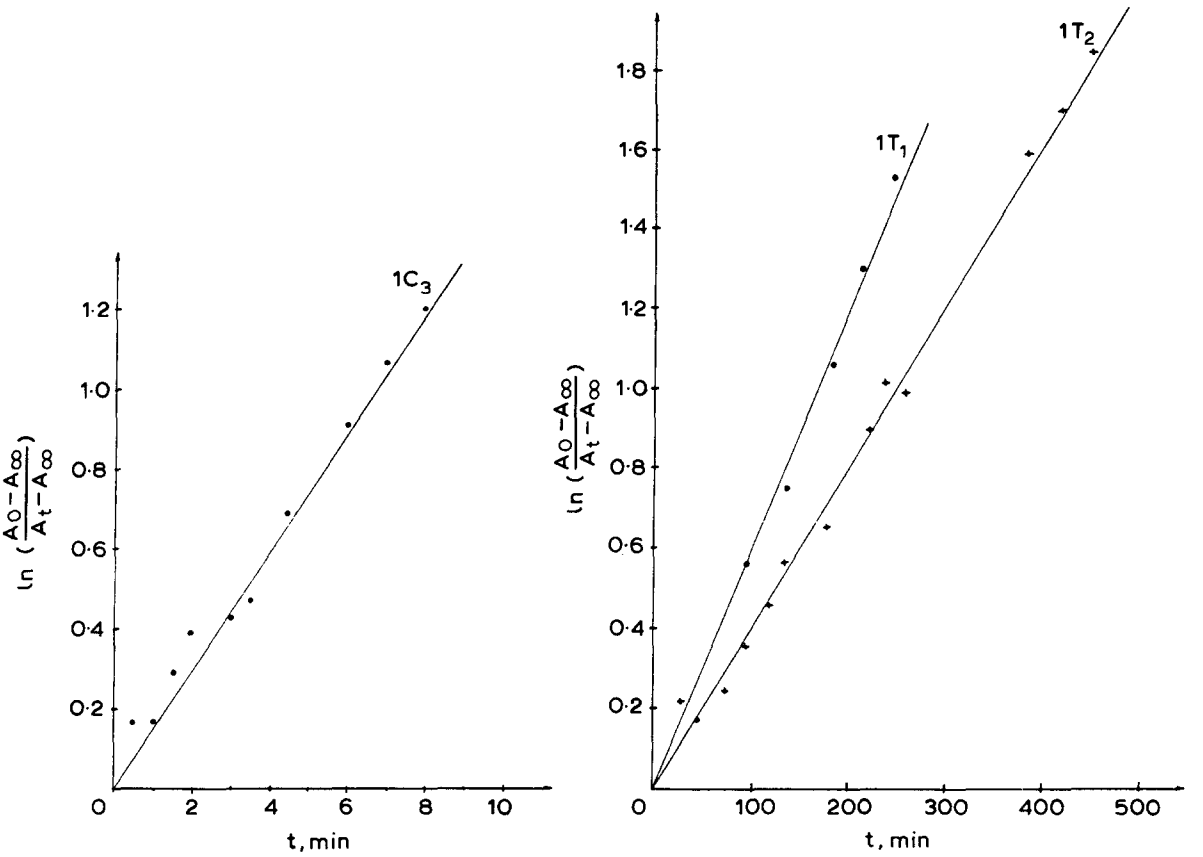

Fig. 1. Determination of the rate constant of the cyclization reaction for carrageenans $1C_3$, $1T_1$ and $1T_2$.

Table 2. Rate constants and half-lives of the cyclization reaction in carrageenans $1C_3$, $1T_1$, and $1T_2$

Temperature (°C)	k ($\times 10^4$ s^{-1})			$t_{1/2}$ (min)		
	$1C_3$	$1T_1$	$1T_2$	$1C_3$	$1T_1$	$1T_2$
35	0·46	—	—	252	—	—
50	2·3	—	0·04	49·9	—	3000
60	4·8	—	0·14	24·0	—	850
70	13·0	0·43	0·35	9·0	268	320
80	26·0	0·98	0·67	4·5	117	170
90	59·0	2·62	1·11	2·0	44·0	103

Table 3. Influence of the concentration of alkali on the cyclization reaction at 60°C

NaOH (M)	k ($\times 10^4$ s^{-1})		$t_{1/2}$ (min)	
	$1C_3$	$1T_2$	$1C_3$	$1T_2$
1·0	4·8	0·14	24	850
1·5	7·8	0·31	15	374
2·0	10·4	0·54	11	216
3·0	15·7	0·90	7·4	128
4·0	26	1·48	4·5	78
5·0	—	1·59	—	73
6·0	31	2·17	3·7	53

Table 4. Influence of the ionic strength on the cyclization reaction at 80°C

I	k ($\times 10^4$ s^{-1})		$t_{1/2}$ (min)	
	$1C_3$	$1T_2$	$1C_3$	$1T_2$
0·5 (0·5 M NaOH)	8·3	0·33	14·0	349
1·0 (0·5 M NaOH, 0·17 M Na_2SO_4)	13·8	0·47	8·4	245
1·0 (1·0 M NaOH)	26	0·67	4·5	172

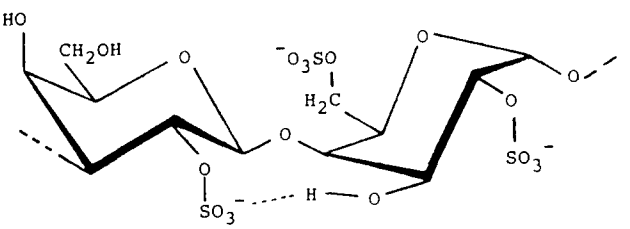

Fig. 2. Proposed interaction between the sulfate on C-2 of the β-unit and the hydroxyl on C-3 of the α-unit.

the clustering of the sulfate groups around the hydroxyl on C-3 of the α-unit shield it from polarization or ionization, reducing the cyclization reaction rate. Furthermore, as lambda-carrageenans carry a sulfate group on C-2 of the β-unit, another effect is produced

by interaction of this sulfate with the hydroxyl group on C-3 of the α-unit (Fig. 2). Molecular models suggest an appropriate geometry for this interaction which has also been reported for heparin sulfates (Fransson *et al.*, 1980). This effect could explain why the second-order rate constant increases in lambda-carrageenans to a certain value with the concentration of alkali, while in carrageenans of the kappa-family it remains constant (Table 3). This hypothesis is reinforced by the observation that, after autohydrolysis of $1T_2$, the ^{13}C-NMR spectrum shows a displacement of the signal for C-1 of the α-unit from 94·7 ppm to 94·2 ppm; this shift is probably due to the hydrolysis of the sulfate on C-2 of the β-unit (Noseda & Cerezo, in press).

The α-units in carrageenans of the kappa-family are 6-sulfated and 2,6-disulfated; in lambda-carrageenans these units are only 2,6-disulfated. Only one pseudo first-order rate constant was obtained for the cyclization reaction of $1C_3$ (Fig. 1) in spite of the presence of α-D-galactose-6-sulfate units together with the disulfated residues (Table 1). It is not known whether this result is due either to (1) similar reaction rates for the cyclization of the 4-linked galactose 6-sulfate and 2,6-disulfate units caused possibly by the balance of opposite effects in these last residues (the repulsion between the oxygen on C-3 and the sulfate group on C-2 of the α-unit would favor the change of conformation, but this new arrangement would place the latter group in the axial position); or (2) the amounts of the α-D-galactose-6-sulfate units (these are too small to be detected).

In spite of the fact that both nuclear phases of the Gigartinaceae contain a sulfohydrolase which converts 4-linked galactose 6-sulfate and 2,6-disulfate units to the corresponding 3,6-anhydrogalactose residues, this reaction only takes place with mu- and nu-precursors in haploid plants. This enzyme is inhibited by lambda-carrageenans, suggesting that sulfation on C-2 is deleterious (Wong & Craigie, 1978). On the basis that the sequential steps of the in-vivo cyclization are similar to those of the chemical one, the previously mentioned clustering of the sulfate groups around the hydroxyl on C-3 of the α-unit and the interaction between the sulfate group on C-2 on the β-unit and the hydroxyl group on C-3 of the α-unit (Fig. 2) would explain why cyclized derivatives of lambda-carrageenans are not found in nature.

The ease with which the cyclization reaction takes place for kappa/iota- and mu/nu-carrageenans indicates that the alkaline treatments used industrially to increase the gelling properties of crude carrageenans could be carried out under milder conditions giving products with higher gel strengths and lower degradation. Besides, extraction of carrageenans under mild alkaline conditions may produce cyclization, even at low temperatures; therefore, analysis of the system should take into account that the products under study could be different from the native ones.

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