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Phosphorylated polysaccharides. 3. Synthesis of phosphorylated curdlan and its polyelectrolyte behaviour compared with other phosphorylated polysaccharides

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ABSTRACT

A water-soluble phosphorylated curdlan (PCurd) was synthesized by reaction of curdlan with phosphorous acid in molten urea. The structure of PCurd was investigated by FTIR and NMR spectroscopy and the substitution degree was calculated from potentiometric titration. It was found that the obtained curdlan derivative is a monobasic curdlan phosphate with –HP(O)(OH) groups. The polyelectrolyte behaviour of PCurd, studied by electrochemical methods and by viscometry, was compared with other phosphorylated polysaccharides. A slight influence of the polysaccharide chain flexibility on the polyelectrolyte dissociation was observed.

1. Introduction

Curdlan, a bacterial polysaccharide formed by pure culture fermentation of *Agrobacterium biobar 1* (identified as *Alcaligenes faecalis var. myxogenes*) is a polysaccharide with a linear structure composed entirely of D-glucose linked by β-glucosidic bonds in (1 → 3) positions (Laroche & Michaud, 2007). Water insolubility of curdlan, generally attributed to the existence of extensive intra/intermolecular hydrogen bonds, limited the biological applications. This disadvantage can be eliminated by derivatization reactions of curdlan. The derivatives of curdlan with sulphate (Wang, Zhang, Yu, & Cheung, 2009; Yoshida, Hatanaka, & Uryu, 1990), carboxymethyl (Jin, Zhang, Yin, & Nishinari, 2006), and ammonium groups (Borjihan, Zhong, Baigude, Nakashima, & Uryu, 2003; Numata, Sugikawa, Kaneko, & Shinkai, 2008) were reported. The data concerning the synthesis and characterization of water-soluble phosphorylated derivatives of curdlan are rather scarce. Only a phosphate derivative obtained by reaction of curdlan with phosphoric acid in dimethyl sulphoxide – urea was presented in the literature (Chen, Xu, Zhang, & Zeng, 2009; Williams et al., 1991). In recent years, many papers have reported, for native curdlan and its derivatives, an important biological activities such as anti-tumour, anti-HIV (Borjihan et al., 2003; Yoshida et al., 1990), anti coagulant (Alban & Franz, 2001), anti-oxidant activity and immunomodulatory effects (Chen & Seviour, 2007; Laroche & Michaud, 2007).

In this study, we present the synthesis, characterization and properties of water-soluble phosphorylated curdlan obtained by reaction with phosphorous acid in molten urea. The polyelectrolyte behaviour of the curdlan derivative was compared with other phosphorylated polysaccharides (dextran and cellulose).

2. Experimental

2.1. Materials

Curdlan (*Alcaligenes faecalis*) from Wako Pure Chemical Ind., Japan was kindly provided by Prof. T. Uryu. The reagents: phosphorous acid 99% from Sigma–Aldrich, Germany, urea 99.5% from Carl Roth, Germany, and methanol from Chimopar, Romania, were used without further purification. The monobasic dextran phosphate (PDex, with degree of substitution about 1.5), and the monobasic cellulose phosphate (PCel, with degree of substitution about 1) were synthesised in our laboratory (Suflet, Chitanu, & Desbrières, 2010; Suflet, Chitanu, & Popa, 2006).

2.2. Methods

2.2.1. Synthesis of phosphorylated curdlan

Phosphorylation of curdlan was performed by reaction of curdlan with phosphorous acid (Inagaki, Nakamura, Asai, & Katsuura, 1976). To obtain the reaction media, 20 g urea was heated at 140 °C into a flask equipped with nitrogen inlet, condenser, and stirrer. 2 g curdlan (Curd) and 10 g phosphorous acid were added portion wise alternatively to the molten urea. The temperature was raised to

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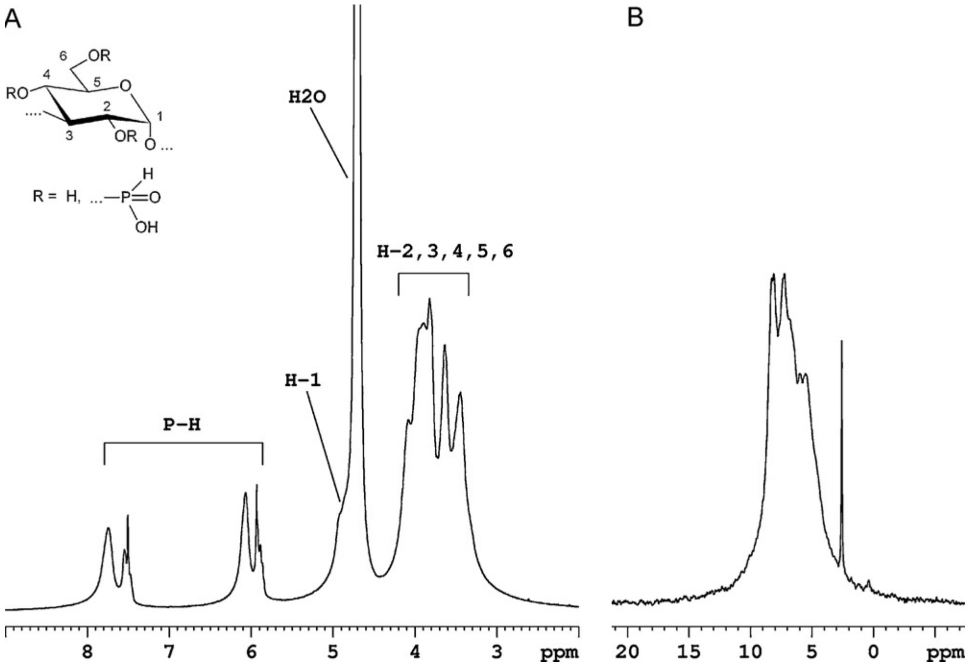

Fig. 1. NMR spectrum of phosphorylated curdlan: (a) ^1H NMR and (b) ^{31}P NMR decoupled.

145 °C and the reaction was allowed to proceed for 3, 4 or 5 h. After cooling, the reaction mixture was dissolved in 1 N NaOH aqueous solution and precipitated in methanol. The derivative was purified by several reprecipitations and dried under reduced pressure for 48 h. The final product, PCurd in the sodium salt form, is a white powder soluble in water. The advanced purification of PCurd was performed by diafiltration in a Millipore cell with PM10 membrane. The diafiltration was stopped when the filtrate conductivity was lower then 5 μS/cm and the polymer was recovered from aqueous solution by freeze-drying.

2.2.2. *Analysis and characterization of phosphorylated curdlan*

Elemental analysis of phosphorous was estimated by EDX analysis performed on the Quanta 200 (FEI) electron microscope equipped with EDX system. FTIR spectra were recorded on KBr pellet using a Vertex 70 Bruker spectrometer. The NMR spectra have been recorded on a Bruker Avance III 400 spectrometer, equipped with a 5 mm multinuclear inverse detection probe, operating at 400.1 and 161.9 MHz for ^1H and ^{31}P nuclei, respectively. ^1H chemical shifts are reported in δ units (ppm) relative to the residual peak of the solvent (ref.: H_2O, ^1H, 4.8 ppm). The molar mass of PCurd was estimated by size exclusion chromatography using a Waters instrument equipped with two Ultrahydrogel columns (250 and 2000, 7.8 × 300 mm) and a refractive index detector using aqueous 0.1 M $NaNO_3$ solution with 3% acetonitrile as eluent and poly(acrylic acid) samples as standard. The decomposition under dynamic conditions of heating has been investigated with a Paulik–Paulik–Erdey MOM-Budapest instrument on 50 mg samples, at 12 °C/min, in air.

Polyelectrolyte behaviour was studied by electrochemical methods and by viscometry. Potentiometric titration was performed using a Metrohm all purposes 716 DMS Titrino apparatus equipped with a 6.0203.100 combined electrode. A 712 Metrohm conductometer with 6.0908.110 cell was used for the conductometric titrations. The potentiometric and conductometric titrations were made on the acid form of phosphorylated polysaccharides. For this, diluted solutions of phosphorylated polysaccharides in Na salt were passed through a cationic exchange column filled with a sulfonic Dowex 50 W × 8, 20–50 mesh resin. Viscometric measurements were performed with an Ubbehlode viscometer with type 0a capillary, at 25 ± 0.02 °C using an AVS 350 Schott automatic viscosity measuring system.

3. Results and discussion

3.1. *Chemical structure*

The degree of substitution (DS) of PCurd was evaluated by potentiometric or conductometric titration according to the methods already verified (Suflet et al., 2006) and compared with values obtained by EDX analysis. The value of DS was 1 ± 0.11 regardless of the reaction time.

In the FTIR spectrum of curdlan the following characteristic bands are observed: a large band at 3400 cm^{-1} corresponding to OH groups; a band at 2915 cm^{-1} attributed to CH_2 groups; characteristic band at 1652 cm^{-1} owing the associated water; bands 1436–1262 cm^{-1} for CH_2 and C–OH from the glucosidic units; a large band at 1164–993 cm^{-1} for C–O–C from the glucosidic units and a band at 889 cm^{-1} for β-D-glucosidic bonds (Yang & Zhang, 2009). In the FTIR spectrum of PCurd several new bands appeared: a band at 2410 cm^{-1} corresponding to the P–H bond, another one at 1216 cm^{-1} corresponding to the P=O bond, a shoulder at 1054 cm^{-1} attributable to the P–OH bond, and a band at 836 cm^{-1} corresponding to the P–O–C bonds (Hesse, Meier, & Zeeh, 1984).

The NMR data provide evidence for the formation of phosphorylated curdlan. In the ^1H NMR spectrum of PCurd, presented in Fig. 1a, two peaks at 6.17 and 7.84 ppm are observed which correspond to a doublet with a coupling constant J_{H-P} of 668 Hz. This value is characteristic for the P–H bond. The presence of the other two peaks at 7.65 and 5.99 ppm indicate that $-H_2PO_3$ groups occupied not only the C-6 position in glucosidic unit, but also C-2 or C-4 positions. The two sharp peaks observed at 7.60 and 6.03 ppm can be assigned to small traces of unreacted H_3PO_3.

The ^{31}P totally decoupled NMR spectrum of PCurd, presented in Fig. 1b, exhibits a broad and complex signal between 2 and 12 ppm. It can be assigned to the phosphorus atoms from the $-H_2PO_3$ groups, present in different positions in the glucosidic units. The sharp signal at 2.6 ppm corresponds to the free H_3PO_3 which remained entrapped. These data in association with FTIR

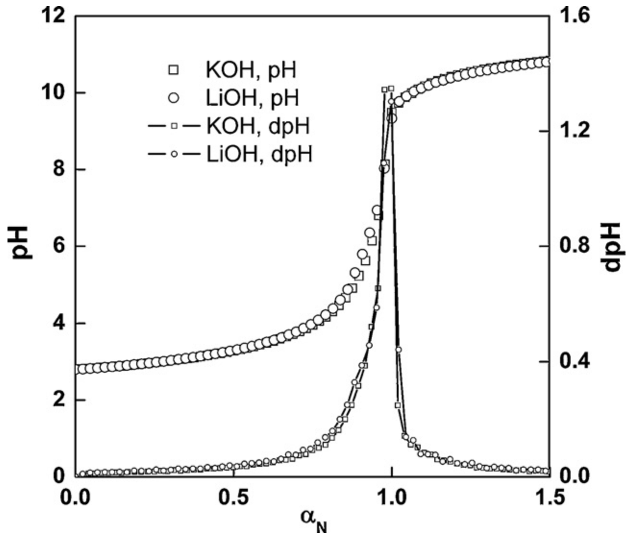

Fig. 2. Potentiometric titration of PCurd with monovalent bases without added salt.

spectra (band at 2405 cm^{-1}, corresponding to the P–H bond) classifying our phosphorylated curdlan as a curdlan phosphate, namely monobasic curdlan phosphate.

The phosphorylation was performed in quite severe conditions when the destruction of the polysaccharide chain could be expected. In spite of that, the molar mass of phosphorylated curdlan, determined by size exclusion chromatography in aqueous salt solution was about 178,000 g/mol. Also, thermogravimetric analysis of native curdlan has revealed a maximum decomposition temperature of 280 °C, corresponding to the maximum rate of weight loss.

3.2. Polyelectrolyte behaviour

3.2.1. Potentiometric titration

The removal of electrolyte impurities is important in polyelectrolyte behaviour. Therefore the reprecipitation followed by diafiltration was repeated three times and only then the phosphorylated polysaccharides were transformed to the acid form and used for titrations. Aqueous solution of phosphorylated polysaccharides (PCurd, PCel, and PDex) in acid form was titrated with monovalent bases (0.1 N KOH and 0.1 N LiOH) at different ionic strengths. The concentration of the polyelectrolyte solutions was 3.2×10^{-3} equi./L. The added salt, KCl and LiCl, used at the titration with KOH and LiOH, respectively, was in concentration from 10^{-2} up to 1 M. In Fig. 2 are presented the potentiometric titration curves of PCurd with monovalent bases in the absence of added salt and their first derivates.

All the titration curves present only one inflexion point, corresponding to the dissociation of one kind of acidic groups –HP(O)(OH). In the limit of the experiments, no selectivity between Li$^+$ and K$^+$ was observed. The presence of salt in the system (Fig. 3) has a slight influence on the polyion dissociation in according to the case of strong polyelectrolytes (Nagaya, Minakata, & Tanioka, 1999). The potentiometric titration curves of PCel are similar. From potentiometric data the apparent dissociation constant, pK_a, have been calculated using the Henderson–Hasselbalch relation:

$$\mathrm{p}K_a = \mathrm{pH} + \log\left(\frac{1-\alpha_T}{\alpha_T}\right) \tag{1}$$

where pK_a is the apparent dissociation constant; $\alpha_T = \alpha_{H^+} + \alpha_N$ is the total fraction of ionized groups, α_{H^+} is the initial degree of dissociation, and α_N is the degree of neutralization. The intrinsic dissociation constant pK_0 was estimated from extrapolation of pK_a = $f(\alpha_T)$ curves. In Table 1 are presented the pK_0 values obtained from the potentiometric titrations with monovalent bases of PCurd and PCel compared with PDex. It can be seen that the order of pK_0 values is in accordance with the flexibility parameter S resulting from the viscometric measurements (col. 4, see Section 3.2.3), suggesting that the dissociation is influenced by the accessibility of the anionic groups. The values of pK_0 are in accordance with the values given in the literature for polyelectrolytes with moderately strong acid groups (Minakata, Takayama, Yano, Tanaka, Ariki, & Shimizu, 2003), but higher that the first dissociation constant of the diprotic phosphorous acid (p$K_0^1 = 1.3$) (Lide, 2003–2004).

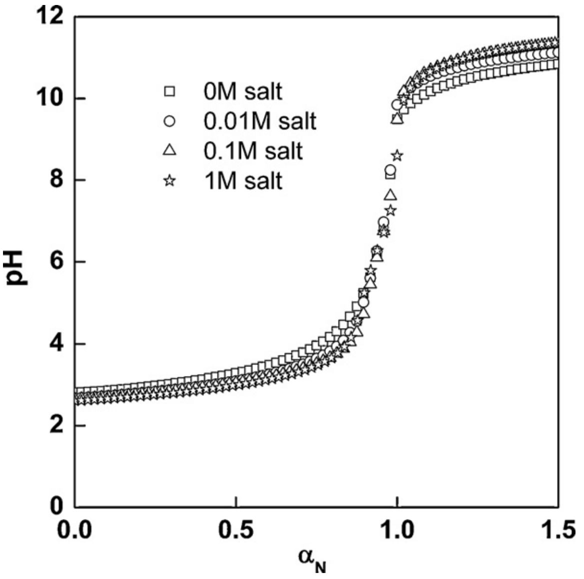

Fig. 3. Influence of added salt on the potentiometric titration of PCurd with KOH.

3.2.2. Conductometric titration

The conductometric titration curves of PCurd and PCel obtained at the neutralization with monovalent counterions are given in Fig. 4a. The curves have the "V" shape, well-known for strong polyacids with monobasic acid groups (Dautzenberg et al., 1994). The sharp decrease of the conductivity in the first part of the titration curves is related to the neutralization of the completely dissociated H$^+$ ions (with a high equivalent ionic conductivity) from the polyelectrolyte with the OH$^-$ ions from the added base. After the equivalence point, the increase of the conductivity is due to the excess of added base. In Fig. 4b the conductometric titration curves of PDex was showed. In this case, DS was 1.5 and therefore the curves present two inflexion points corresponding to the dissociation of two kinds of monobasic acid groups attached in different positions of the glucosidic unit (Suflet et al., 2010).

The transport coefficient of monovalent counterions (f^{M^+}) and the equivalent conductance of polyanion λ_p can be obtained from

Table 1
The dissociation constants pK_0 for phosphorylated polysaccharides.

Sample	KOH	LiOH	S
PCurd	2.81	2.79	0.799
PCel	2.41	2.42	0.053
PDex	2.60	2.60	0.139

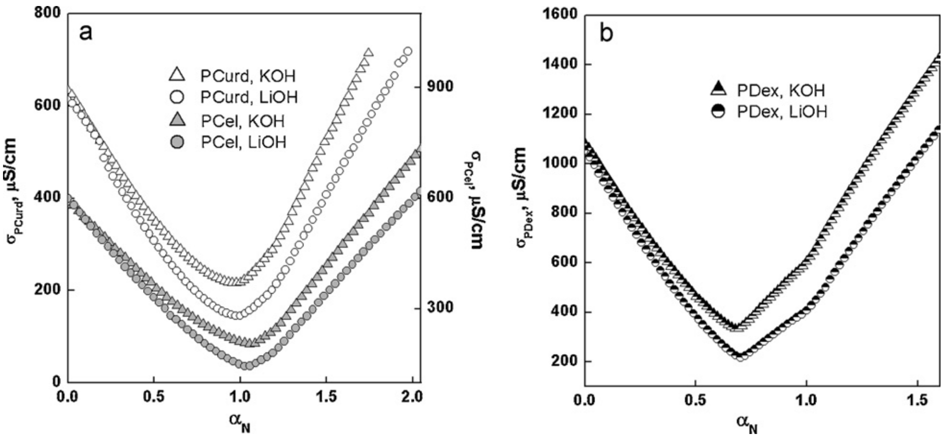

Fig. 4. Conductometric titration of PCurd and PCel (a) and of PDex (b) with monovalent bases.

the conductometric data using the relations (2):

$$f^{M^+} = \frac{\sigma_{K^+} - \sigma_{Li^+}}{10^{-3}C_P(\lambda_{K^+} - \lambda_{Li^+})}$$
$$\lambda_P = \frac{\sigma_{Li^+} - 10^{-3}C_P f^{M^+}\lambda_{Li^+}}{10^{-3}C_P f^{M^+}} \tag{2}$$
$$f^{H^+} = \frac{\sigma_i}{10^{-3}C_P\lambda_{H^+}}$$

where σ_{Li^+} and σ_{K^+} (μS) are the conductivities at $\alpha_N = 1$ by neutralization with KOH or LiOH; σ_i is the conductivity of polyelectrolyte solution at the beginning of titration; C_P is the polymer concentration (equi./L), and λ_{H^+}, λ_{Li^+}, λ_{K^+}, and λ_P ($cm^2\ \Omega^{-1}$ equi.$^{-1}$) are the equivalent conductivities of H^+, Li^+, K^+ ions and of polyion, respectively. f is defined as the ratio of the self-diffusion coefficients of the counterion in polyelectrolyte solution and in pure solvent, respectively, and can be assimilated with the fraction of free counterions (Milas, Shi, & Rinaudo, 1990). In Table 2 are presented the transport coefficients of the monovalent counterions and the polyion equivalent conductivity calculated from conductometric data, at complete neutralization. The values α_{H^+} and f^{H^+} calculated from potentiometric and conductometric data, respectively, are comparable. The values of f^{M^+} and λ_p are comparable to those found for other ionic polysaccharides as polygalacturonic acid or alginic acid (Arguelles-Monal, Cabrera, Peniche, & Rinaudo, 2000; Thibault & Rinaudo, 1985; Tuffile & Ander, 1975). The transport parameter of monovalent ions indicates that PCurd has the greater degree of dissociation.

3.2.3. *Viscometric behaviour*

Viscometric measurements were performed on phosphorylated polysaccharides without or with added salt in concentration from 10^{-3} up to 1 M. For example, in Fig. 5 is presented the dependence $\eta_{sp}/C_P = f(C_P)$ of PCurd in the absence or in the presence of KCl.

All the three phosphorylated polysaccharides exhibit typical polyelectrolyte behaviour. With no added salt, the reduced viscosity increased sharply with decreasing polymer concentration because in high diluted solutions the charges induce a fully stretched conformation of macroions. By increasing the amount of added salt, the electrostatic repulsions are gradually screened, so that at high salt concentration the macroion behaves as neutral polymer (Cohen & Priel, 1989; Cohen, Priel, & Rabin, 1988; Mandel, 1987). The Fuoss relation (3) was used to evaluate the intrinsic viscosity of phosphorylated polysaccharides.

$$\frac{C_P}{\eta_{sp}} = \frac{1}{A} + \left(\frac{B}{A}\right) C_P^{1/2} \tag{3}$$

where A is the intrinsic viscosity and B is a constant (Dragan, Mihai, & Ghimici, 2003; Fuoss & Cathers, 1949; Strauss & Smith, 1953; Wolf, 2007; Ydens, Moins, Degee, & Dubois, 2005). In Fig. 6 shows the Fuoss plot of PCurd at various concentration of added salt.

The decrease of the intrinsic viscosity with the increase of the ionic strength may be expressed by the relation (Dautzenberg et al., 1994; Malovikova, Milas, Rinaudo, & Borsali, 1993, chap. 24; Pals & Hermans, 1952):

$$[\eta]_{C_S} = [\eta]_\infty + SC_S^{-1/2} \tag{4}$$

where C_S is the added salt concentration (mol/L), $[\eta]_\infty$ is the intrinsic viscosity (g/dL) extrapolated at infinite ionic strength, and the slope S is related to the chain stiffness and expansion. From Fuoss plots, the intrinsic viscosity at different concentrations of

Table 2
f parameters and polyion equivalent conductivity of phosphorylated polysaccharides.

Sample	f_{M^+}	f_{H^+}	α_{H^+}	λ_p
PCurd	0.57	0.50	0.51	31.64
PCel	0.49	0.46	0.50	40.67
PDex	0.52	0.40	0.43	54.70

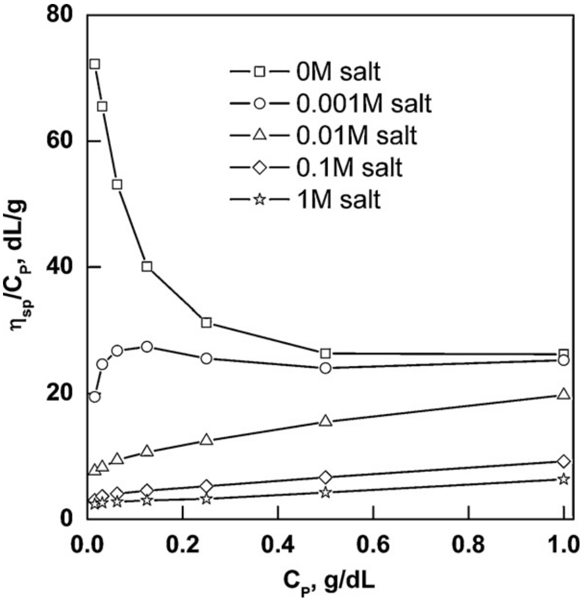

Fig. 5. η_{sp}/C_P dependence on polymer concentration at various ionic strengths of PCurd in the absence or in the presence of KCl.

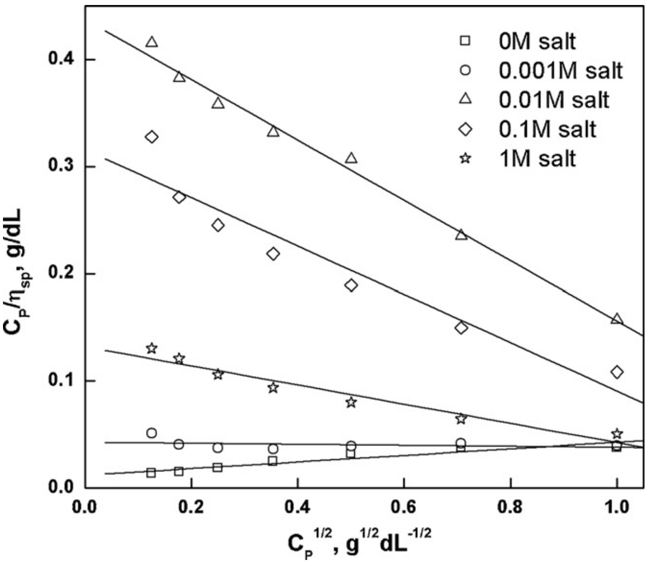

Fig. 6. C_P/η_{sp} dependence on the $C_P^{1/2}$ of phosphorylated curdlan at various concentrations of added salt (KCl).

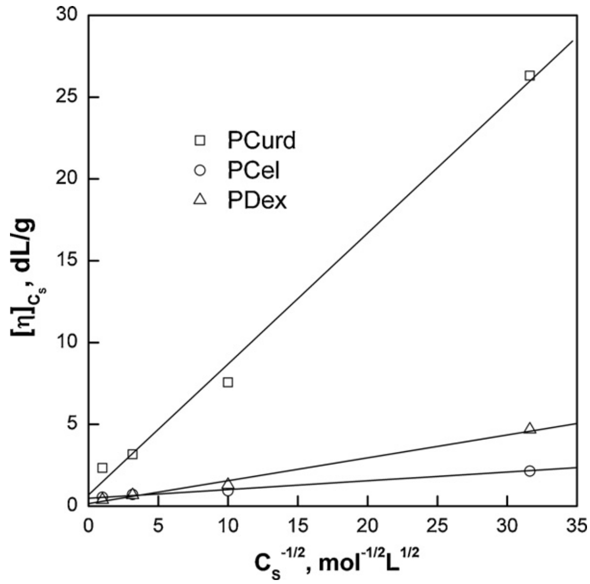

Fig. 7. Dependence of intrinsic viscosity of phosphorylated polysaccharides on added salt concentration.

added salt was determined and plotted as function of $C_S^{-1/2}$, according to relation (4), as shown in Fig. 7.

In Table 3 are gathered the viscometric parameters $[\eta]_{\text{water}}$, $[\eta]_\infty$, and S. The value of $[\eta]_{\text{water}}$ was obtained from the extrapolation of Fuoss equation (3) at infinite dilution in aqueous solution without added salt. $[\eta]_\infty$ is the intrinsic viscosity value at infinite salt concentration, obtained with Eq. (4). The parameter S is calculated with the relation (4) and it is a measure of the chain flexibility. This parameter seems to influence the dissociation constant of the acid groups, as shown in Table 1. From these data we can estimate the role of the linkage of the glucopyranosic units from the polysaccharide chain on the derivatives flexibility and on their polyelectrolyte behaviour. PCel and PCurd have almost the same molecular mass and the $[\eta]_\infty$ values are not very different, but PCel is the most rigid one, probably because of the β-(1 → 4) glucosidic linkage that determine the supramolecular structure.

Table 3
Viscometric parameters for the studied phosphorylated polysaccharides.

Sample	$[\eta]_{\text{water}}$ (dL/g)	$[\eta]_\infty$ (dL/g)	S	Molar mass (g/mol)
PCurd	81.93	0.69	0.799	178,000
PDex	18.72	0.15	0.139	324,000
PCel	4.59	0.48	0.053	180,000

4. Conclusion

Phosphorylated curdlan was synthesized by reaction of curdlan with phosphorous acid in molten urea. Fully water-soluble derivatives were obtained with degree of substitution up to 1, for reaction times of 3, 4, and 5 h. FTIR spectra, electrochemical titration, and NMR spectra confirmed that the reaction between curdlan and H_3PO_3 takes place with formation of curdlan monobasic phosphate Curd –O–HP(O)OH. The polyelectrolyte behaviour was investigated by potentiometric and conductometric titrations with monovalent bases in presence or absence of low molecular added salt and by viscometric measurements. The potentiometric curves display one inflexion point corresponding to the dissociation of one acid group. The addition of low molecular weight salts has a slight influence on the polyion dissociation. The conductometric titration evidenced the dissociation of the phosphorylated polysaccharides as strong polyacids. From viscosity measurements of phosphorylated curdlan, cellulose, and dextran, the stiffness parameters were obtained. The higher flexibility of PCurd can be due to the type of linkage of D-glucose units in the polysaccharide chain. A slight influence of the flexibility parameter on the polyelectrolyte dissociation was evidenced.

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