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One-step introduction of amine and ammonium groups and cross linking of polygalacturonic acid

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Polygalacturonic acid (PGA) was cross linked with epichlorohydrin (E), E-NH_4OH and also with E-NH_4OH in the presence of 3-chloro-2-hydroxypropyl-trimethylammonium chloride (CHMAC). The reactions were optimized with the goal being to obtain the maximal yield and nitrogen content of insoluble residue (IR) or water-soluble fraction (WSF). Also the amount of nitrogen incorporated into IR or WSF from NH_4OH and/or CHMAC used was considered for evaluation of the optimal conditions. In the absence of E and NH_4OH, bad yields of quaternized PGA WSP were obtained, indicating the degradation of PGA in the absence of a cross linking agent. The presence of functional groups was confirmed by ^{13}C-solid state NMR in IR, as well as by NMR in solution for WSF. The linkage of cross linked PGA to functional groups, or the degree of cross linking, could not be estimated from the NMR results. This we believe is because the rigidity of the cross linked matrix which causes the absence of PGA signals in the solid-state NMR spectrum. © 1997 Elsevier Science Ltd

INTRODUCTION

Pectin has the potential to become a source for preparation of gel-forming, ion-exchanging, dye-binding and chelating materials. The obtained results indicate that after cross linking it could be used for the separation of proteins, enzymes and cations (Hatanaka *et al.*, 1990; Vajayalakshima *et al.*, 1978; Kohn *et al.*, 1976; Inoue *et al.*, 1984; Tibenský, 1968; Rexová-Benková & Tibenský, 1972; Tibenský & Kuniak, 1972). Also water-soluble amidated pectins are used for the study of interaction with calcium ions (Racape *et al.*, 1989). In this study, we have introduced tertiary and quaternary groups to PGA by cross linking in the presence of NaOH and water with E-NH_4OH, E-CHMAC and E-NH_4OH-CHMAC. This could be schematically ascribed by the reaction equation:

$$NH_3 + E + CHMAC + HO{-}PGA{-}COONa + OH^- + H_2O \rightarrow R{-}O{-}PGA{-}O{-}[CH_2CH(OR)CH_2N(CH_2CH(OR)CH_2)_2]_n{-}O{-}PGA{-}COOR + OH^- + H_2O$$

where R could be hydrogen, sodium, trimethylammoniumhydroxypropyl (TMAHP) or hydroxypropyl (HP) group. The prepared derivatives were characterized by NMR in D_2O, solid-state NMR and elemental analysis. The goal was to prepare water-soluble, as well as insoluble, derivatives and to compare the obtained data with the results on starch (Šimkovic, 1996). The use of PGA for preparation of ion-exchangers has an advantage in comparison to starch, because it already contains carboxyls, and by introduction of ammonium and amino-groups one could expect to prepare an ion-exchanger with amphoteric properties.

EXPERIMENTAL

Materials

PGA (Fluka, reg. no. 81325, 25–50kDa), CHMAC (50% solution, Spolek pro chemickou a hutní výrobu, Ustí nad Labem, Czech Republic or 60% solution, Serva) and all the chemicals used were commercial grade.

Methods

^{13}C solid-state and ^1H-and ^{13}C-NMR spectroscopy in D_2O, and all other methods used were described previously (Šimkovic *et al.*, 1996).

Preparation of samples

PGA (1.74g, 10mM) was mixed with or without E, CHMAC, NH_4OH, NaOH and water as listed in Table

Table 1. Quantities of reactants used for cross linking of PGA in the presence of CHMAC or NH_4OH and results of individual experiments

Sample [#]	Moles of reactants[a]					Yield [%]	N [%]	N[b] [%]
	E	NaOH	NH_4OH	H_2O	CHMAC			
1	0.10	0.10	0	0.500	0	38[c]	0	0
						85[d]	0	0
2	0.25	0.50	0	1.000	0	21[c]	0	0
						252[d]	0	0
3	0.01	0.05	0	0.104	0.01	24[c]	6.88	22
						140[d]	4.13	77
4	0.03	0.05	0	0.313	0.03	73[c]	7.76	25
						241[d]	5.62	60
5	0.03	0.05	0.065	0.378	0	121[c]	4.70	12
						04[d]	0	0
6	0.05	0.20	0.050	0.292	0	63[c]	0.31	1
						52[d]	2.41	3
7	0.05	0.30	0.017	0.100	0	75[c]	0.69	4
						17[d]	2.06	3
8	0.03	0.50	0.065	0.378	0	76[c]	0.33	1
						8[d]	1.76	0
9	0.10	0.10	0.10	0.583	0	48[c]	9.25	6
						363[d]	7.97	38

[a] Same quantity of PGA in all experiments (1.76g; 0.01mol of anhydrogalacturonic acid units).
[b] Nitrogen incorporated from the amount of NH_4OH and/or CHMAC used.
[c] Yield of WSF.
[d] Yield of IR.

1 and stirred for 24h at room temperature. In experiments where NH_4OH was used or water was present only as a component of NH_4OH or CHMAC the solid NaOH was added last and the vial closed to prevent losses of ammonia. The reaction was stopped by washing the mixture into dialysis tubing (12–14kDa MWCO; Sigma) and after exchanging seven volumes of distilled water, separated into IR and WSP. Both components were lyophilized. The yields were calculated on the weight of PGA used on the dry basis.

RESULTS AND DISCUSSION

The quantities of reactants used for the cross linking of PGA are listed in Table 1. When cross linked only with E, NaOH in water at the ratio of 0.1: 0.1: 0.5, the yields of WSP and IR were 38 and 85%, respectively, while at 0.25: 0.5: 1 molar ratio they were 21 and 252% (compare sample 1 and 2). Because NaOH is also consumed by carboxyls, more base was needed than for the cross linking of starch (Šimkovic *et al.*, 1996). In the presence of CHMAC (sample 3), more IR was obtained than with starch (Šimkovic, 1996), under analogical conditions. With increased quantities of E and CHMAC (sample 4), the yields and nitrogen content of both components could be further increased. In the absence of CHMAC, and in the presence of NH_4OH, the WSP decrease with increased amounts of E, NaOH and NH_4OH used (samples 5–9) and at PGA: NaOH: NH_4OH: H_2O = 0.1: 0.1: 0.1: 0.583, resulting in 363% yield of IR (sample 9). At the low concentration of E and NaOH, only WSP could be produced (sample 5). In the presence of E, NH_4OH and CHMAC with a gradual increase of E the quantity of WSP formed decreased, while the yield of IR increased with a constant amount of water, NH_4OH, NaOH and CHMAC (Table 2, compare samples 1–3). A further increase of the quantity CHMAC resulted in the yield decrease of IR (Table 2, compare samples 3 and 4). This indicates that further increase of the quantity of CHMAC does not result in increased yields of IR and quantity of nitrogen incorporated into both fractions. To find out how the quaternization of PGA proceeds in the absence of cross linking agent, E and NH_4OH were omitted. At the ratio of PGA: NaOH: H_2O: CHMAC = 0.01: 0.1: 0.1: 0.01, only 35% yield (N = 1.17%) of WSP could be obtained and at lower concentrations of NaOH, higher concentrations of nitrogen in the WSP at even lower yields (Table 2, compare samples 5–7) were observed.

The ^{13}C-NMR spectrum of the water-soluble part of PGA modified with E (Table 1, sample 1) in D_2O did indicate the presence of both PGA (176.4, C-6; 99.9, C-1; 78.8, C-4; 73.0, C-5; 71.3, C-3; 69.8 ppm, C-2) and hydroxypropyl groups (72.2, 71.8, 71.6, 70.1 and 69.0ppm for CHOH carbons and 63.5ppm for CH_2 groups). When CHMAC was added to the reaction mixture (Table 1, sample 4) only signals of HP and TMAHP groups were observed in WSP fraction by ^{13}C-NMR (68.8, 68.0, 63.6, 62.8, 55.2, 54.7 and 54.0ppm). The absence of PGA signals indicates that only degradation products of E and CHMAC were present in the

Table 2. Quantities of reactants used for modification of PGA and results of individual experiments

Sample [#]	Moles of reactants[a]					Yield [%]	N [%]	N[b] [%]
	E	NaOH	NH_4OH	H_2O	CHMAC			
1	0.01	0.05	0.01	0.128	0.01	169[c]	0.44	5
						0[d]	0	0
2	0.02	0.05	0.01	0.128	0.01	132[c]	4.39	38
						4[d]	2.36	1
3	0.03	0.05	0.01	0.128	0.01	9[c]	4.81	3
						165[d]	5.73	63
4	0.03	0.05	0.01	0.267	0.03	96[c]	2.36	8
						25[d]	3.07	3
5	0	0.10	0	0.100	0.01	35[c]	1.17	5
						0[d]	0	0
6	0	0.01	0	0.100	0.01	21[c]	2.44	7
						0[d]	0	0
7	0	0.05	0	0.070	0.01	19[c]	2.41	6
						0[d]	0	0

[a] Same quantity of PGA in all experiments (1.76g; 0.01mol of anhydrogalacturonic acid units).
[b] Nitrogen incorporated from the amount of NH_4OH and/or CHMAC used.
[c] Yield of water-soluble part.
[d] Yield of insoluble residue.

WSP fraction under these conditions. This is also evident from the extremely high nitrogen content of the sample (7.76%). In one experiment where all the polysaccharide components obtained by the reaction were water-soluble (Table 1, sample 5) and PGA was modified with E and NH_4OH in the absence of CHMAC, three types of HP groups could be expected. According to the ^{13}C-NMR spectrum the signals at 72.2, 71.8 and 69.0ppm might belong to the HP group not linked to NH_4OH, similarly as it was observed in sample 1 (Table 1). For the second type of HP where the group is linked on one end etherifically to PGA and having nitrogen linked on the other end, the signals at 76.8, 65.2, 64.7, 63.7, 62.6 or 60.1ppm might be related. There are additional signals at 55.0, 52.5, 51.3 and 44.1ppm which might belong to HP linked esterifically to PGA carboxyls. WSP fraction cross linked at higher concentrations of E, NaOH and NH_4OH (Table 1, sample 9) did not show the presence of PGA, but only signals in the region from 76.3 to 51.0ppm. These we assigned to poly(hydroxypropylamine) (PHPA), as confirmed by a nitrogen content of 9.25%. So it seems that only in cases where the yields of WSP were close and over 100%, and the nitrogen content from 1 to 5%, the modified polysaccharides could be separated from degradation products. For sample 2 (Table 2) the WSP proved to be, on the basis of ^{13}C-NMR analysis in relation to previous NMR results, a TMAHP-PGA-PHPA derivative (176.4; C-6; 100.0, C-1; 79.0, C-4; 72.4, C-5; 70.0, C-3; and 69.5ppm, C-2 of PGA and 75.5, 74.4, 73.2, 68.7, 66.5, 64.1, 55.8 and 55.3ppm signals of TMAHP and HP groups). The signals at 75.5, 74.4, 66.5 and 64.1ppm were in the minority and according to the results in sample 5 (Table 1), they might belong to HP-amine groups.

The solid-state ^{13}C-NMR of unmodified PGA in the absence of water gives broad signals at 173, 101, 80 and 70ppm, when analyzed by CP experiment. This was close to the data obtained previously (Jarvis & Apperley, 1995). By the addition of 50% of water to the sample, the signals became sharper, but the chemical shifts did not change. As was demonstrated previously (Morgan et al., 1994), the addition of water could help distinguish certain parts or groups in polysaccharides which are not rigid. When the high-power decoupled experiment was used the signals were even sharper (Fig. 1). On IR cross linked with E (Table 1, sample 2) the CP experiment in the presence of water (50% on sample weight) gave signals at 76.6 and 69.4ppm (with shoulders on both sides) and two less intensive signals 50.0 and 43.1ppm. By running the high-power decoupled experiment (Fig. 2) the signals could be better distinguished (78.1, 76.5, 70.8, 69.3, 67.6, 61.4, 50.1 and 43.2ppm). When compared with Fig. 1, it is evident that these sharp signals are not related to the polysaccharide carbons and might belong to the hydroxypropyl bridge of cross linked PGA. There were signals at the same region observed on WSP of this experiment (72.2, 71.8, 71.6, 69.0 and 63.5ppm) with ^{13}C-NMR spectroscopy in solution which we ascribe to hydroxypropyl groups linked just monovalently to PGA. The signals at 50.1 and 43.2ppm might belong to the hydroxypropyl group linked esterifically to carboxyls. It is known that ester groups could be degraded under alkaline conditions, but it is clear that in this way, part of E is consumed and probably not all of them are deesterified. When CHMAC was added to the system (Table 1, sample 4), the CP experiment showed signals at 173.2, 98.7, 67.8, 60.0 and 52.9ppm. Using the high-power decoupled experiment the signals at 66.4, 65.5, 60.4 and 51.6ppm

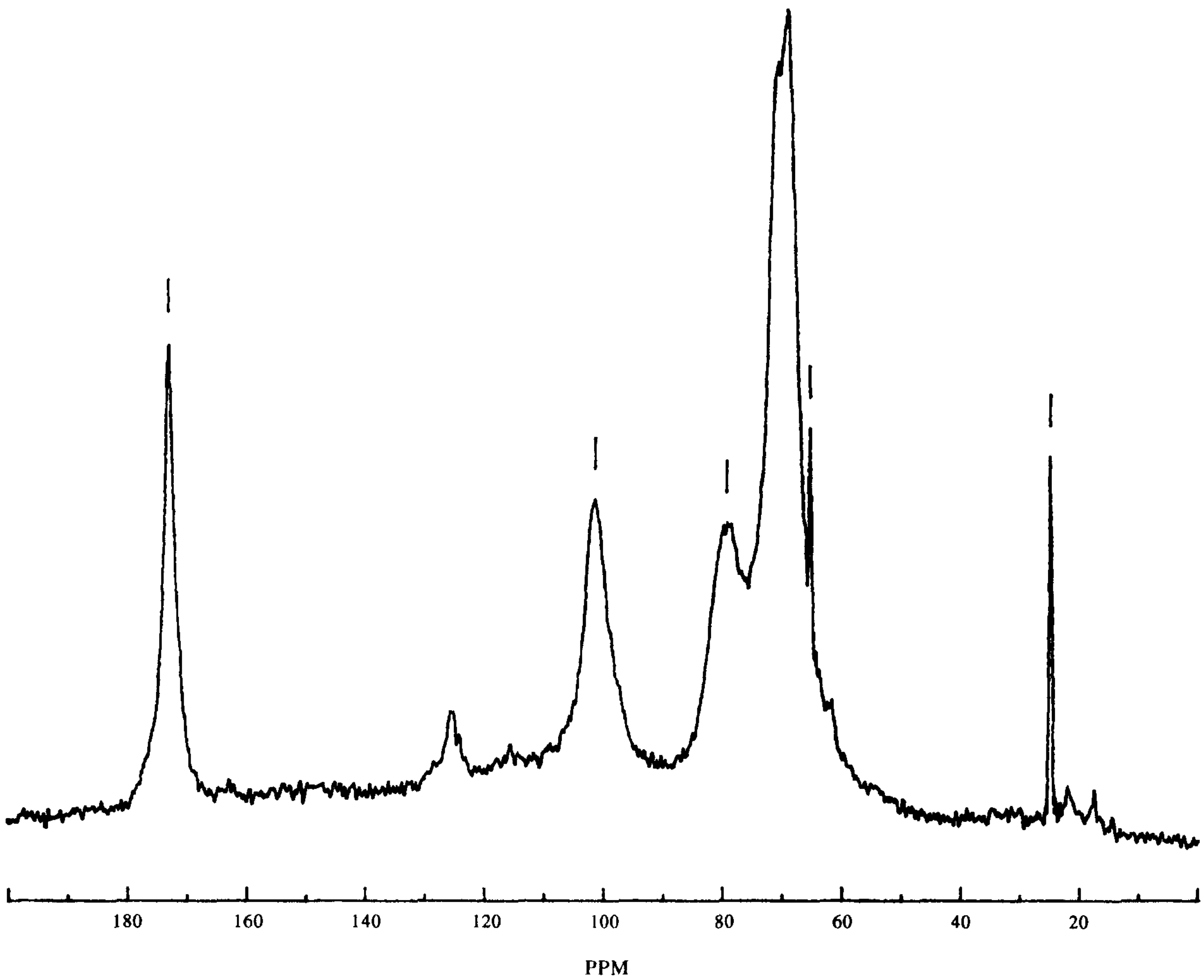

Fig. 1. Solid-state NMR spectrum (high power experiment) of unmodified PGA in the presence of 50% of water.

were observed. The signals at 51.6 and 52.9ppm belong to methyls linked to quaternary nitrogen (Šimkovic, 1996), while the others are related to both types of HP groups. The experiment without CHMAC and with NH_4OH (Table 1, sample 9) gave, on a dry sample, a broad signal at 174, 99, 68 and 62ppm. In the presence of 50% water, sharp signals at 98, 77, 74, 72, 70, 67, 64, 59, 58 and 50ppm were observed by CP experiment. In the high-power decoupled experiment, even sharper signals at 74.0, 70.0, 69.2, 66.9, 66.0, 62.7, 61.2, 60.2, 57.8, 52.5 and 50.1ppm were observed. There were several signals (74.0, 62.7 and 57.8ppm) which were absent in PGA cross linked only with E (Table 1, sample 2). This might belong to the hydroxypropyl groups linked to nitrogen, as was also observed on starch (Šimkovic *et al.*, 1996). Finally, PGA quaternized/cross linked in one step with E, NH_4OH and CHMAC (Table 2, sample 3) showed in dry state by CP experiment broad signals at 174, 99, 68, 60 and 53 ppm. In the presence of 50% water, there were only two signals (67.2 and 38.1ppm), while the high-power decoupled experiment showed three strong signals at 66.0, 60.4 and 52.0ppm and four minor signals at 173.9, 162.6, 97.9 and 43.4ppm. This indicates that the quaternary methyls (52.0ppm) and HPs linked etherically are present, while the CH_2-N are not visible in the spectrum. Similarly as for starch (Šimkovic *et al.*, 1996; Šimkovic, 1996), although the presence of individual groups could be tentatively assigned, the linkage to PGA could not be proven, probably because of the rigidity of the polysaccharide part of cross linked matrix, which made it not visible by this technique. For that reason also, the degree of cross linking in any of the derivatives could not be estimated. The difference in comparison to starch samples is the presence of esterifically linked HP-groups.

CONCLUSIONS

PGA could react with E in the presence of NaOH, NH_4OH, H_2O and CHMAC to produce TMAHP-PGA-PHPA IR, but also WSP with yields dependent upon the ratio of used components. In the absence of NH_4OH or CHMAC, more NaOH and E is needed to obtain good yields of IR. The increased amounts of

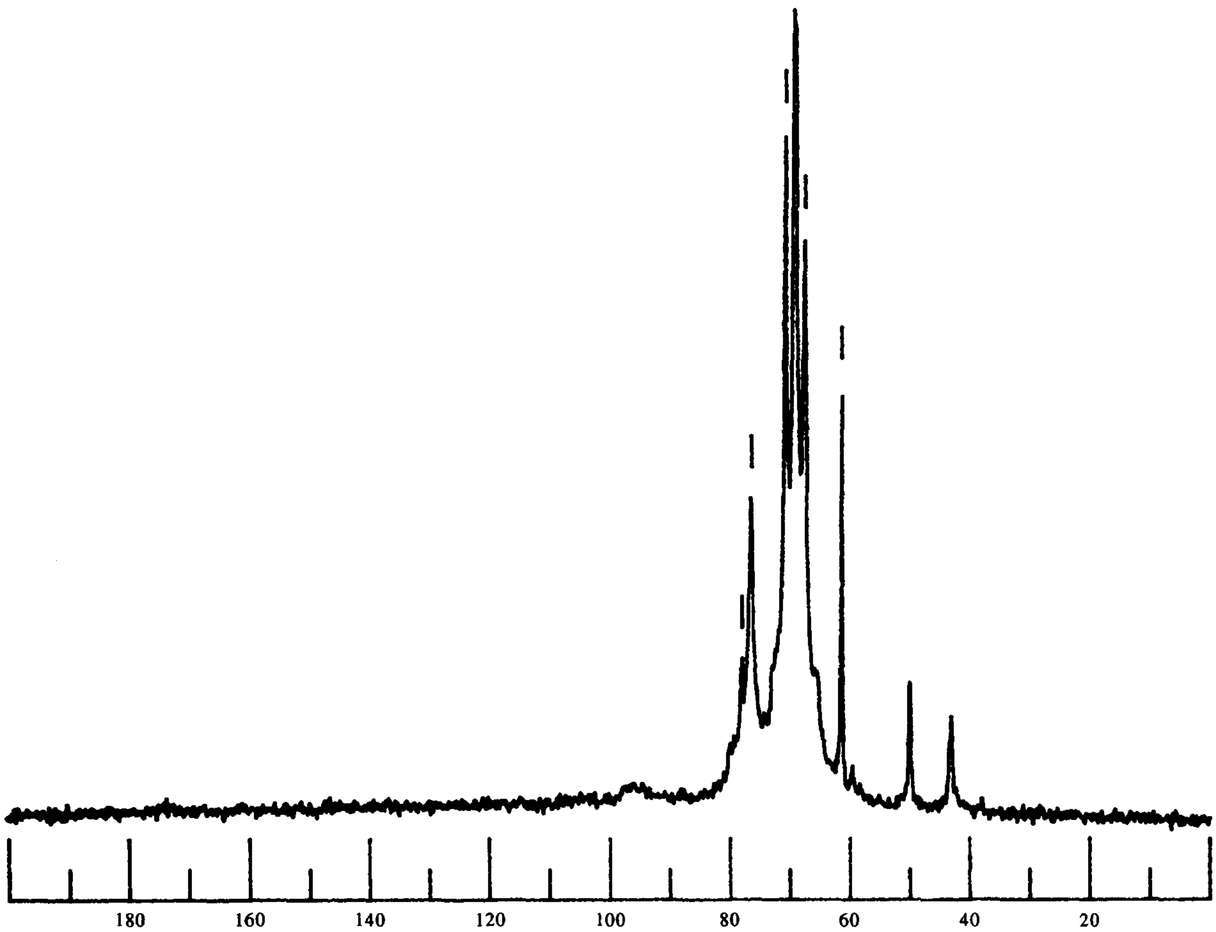

Fig. 2. Solid-state NMR spectrum (high power decoupled experiment) of PGA crosslinked with E (Table 1, sample 2) in the presence of 50% of water.

NH_4OH in the absence of CHMAC causes higher yields of WSP. The amount of nitrogen incorporated into the product from NH_4OH and/or CHMAC was maximally 77% for IR, while it was only 38% for WSP.

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