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EFFECTS OF SOME OXIDISING AGENTS, ESPECIALLY AMMONIUM PEROXYSULFATE, ON SUGAR-BEET PECTINS

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ABSTRACT

Sugar-beet pectins contain feruloyl groups linked to the side chains of the rhamnogalacturonan backbone. Coupling reactions may cross-link these macromolecules. Of several oxidising agents, only hydrogen peroxide–peroxidase and ammonium peroxysulfate were effective, indicating the involvement of free radicals. The effects of peroxysulfate and pectin concentrations, temperature, and the presence of some additives have been investigated mainly by viscometry and chromatography on Sepharose CL-2B. Depending on the concentration of the pectin, the reaction may be used to obtain either water-soluble products of increased molecular weight or gels.

INTRODUCTION

There is interest in the pectins of sugar-beet pulp because of their low cost as raw materials, even though they have poor gelling power due to a high acetyl content[1] and/or a low molecular weight[2,3] as compared to those of apple or citrus pectins.

The fine structure of these pectins has been studied[3,4] using highly purified pectic enzymes and involves side chains of neutral sugars in blocks (so-called hairy regions) along the rhamnogalacturonan backbone, as in pectins from other origins[5–7]. A special feature of sugar-beet pectins is that they contain[3] polyphenols (1–2%) and possibly proteins (3–6%). Among the polyphenols, only ferulic acid residues (~1%) have been proved to be covalently (ester) linked to the hairy fragments of the pectic chain[4]. This substitution does not exist in such other pectins as apple, citrus, cherry, apricot, and potato pectins[8], but has been reported[9] for spinach pectins where the feruloyl residues terminate the neutral-sugar side chains.

Therefore, coupling reactions can be applied to sugar-beet pectins in order to cross-link the molecules. When hydrogen peroxide–peroxidase was used[8], either

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water-soluble products of an increased molecular weight or gels were obtained, depending on the concentration of the pectin. This unusual reaction has been reported only for native water-soluble ferulic acid-containing pentosans (arabinoxylans) from wheat[10,11], and for guaran modified by esterification with ferulic acids[12]. The action of several oxidising agents on wheat pentosans has been investigated and resulted in contradictory conclusions[11,13].

Because the cross-linking reaction may lead to new applications of sugar-beet pectins, their acidic extraction has been extensively studied[2]. We now report on the effects of some oxidants on these pectins as part of a study of the mechanism of the cross-linking reaction and of the properties of the modified pectic molecules.

EXPERIMENTAL

Materials. — Pectin, extracted[2] from sugar-beet pulp at pH 1.0 and 85° for 1 h, contained 0.8% of feruloyl groups and 4% of proteins. The neutral sugar composition as determined below is indicated in Table I. The degrees of methylation and acetylation (mol per mol of uronic acid) were 0.53 and 0.25, respectively[2]. The intrinsic viscosity, measured in 0.155M NaCl, was 138 mL/g, corresponding[14] to a viscosity-average molecular weight of ~30,000.

Apple pectin was a purified commercial sample[15] (Unipectine, Redon, France). Horse-radish peroxidase (EC 1.11.1.7) (type I) was purchased from Sigma, and Sepharose CL-2B from Pharmacia. All reagents were of analytical grade.

Chemical determinations. — The content of galacturonic acid was measured by the *m*-hydroxybiphenyl method[16,17]. The total content of neutral sugars was measured by the orcinol method[18], the values being corrected for interference by uronic acids; the results are expressed in arabinose equivalents. Appropriate calibrations were carried out in order to avoid interference from chemicals and especially from ammonium persulfate which depressed the absorbances even if its concentration was as low as 0.2mM.

TABLE I

CARBOHYDRATE COMPOSITION (MOLAR RATIO TO GALACTURONIC ACID) OF THE INITIAL PECTIN AND OF THE GEL[a] AFTER ELIMINATION OF THE WATER-SOLUBLE PRODUCTS

Component	*Initial pectin*	*Gel*
Galacturonic acid	100	100
Rhamnose	4.9	5.1
Arabinose	3.3	4.6
Xylose	0.8	1.1
Mannose	0.3	0.1
Galactose	19.5	24.0
Glucose	0.5	0.3

[a] 1.2% of pectin; 0.01M ammonium persulfate; 25°.

Individual neutral sugars were quantified by g.l.c. after hydrolysis[19] of the pectins in M H_2SO_4 at 100° for 1.5 h followed by reduction and acetylation of the resulting neutral sugars[20].

The content of feruloyl groups was determined spectrophotometrically from the absorbance at 375 nm of solutions at pH 10.0 (67mM glycine–sodium hydroxide buffer), assuming[21] a molar extinction coefficient of 31,600.

Viscometry. — This was carried out with an automatic Ostwald viscometer (Fica) of ϕ 0.58 mm. Exactly 7 mL of the reaction mixture containing 0.25–1.2% of pectin and chemicals at the desired temperature (±0.01°) were introduced into the viscometer and the flow-times were recorded as a function of time of reaction. Reduced viscosity was calculated and the results are expressed as the ratio of reduced viscosity at time *t* to the initial reduced viscosity.

Viscosities of aqueous 0.25–1.2% solutions of native pectins were also measured as a function of shear-rate (range: 0–128.5 s^{-1}) at 25.00 ±0.05° with a Low-Shear 30 viscometer (Contraves).

Gel-permeation chromatography. — This was performed on a column (1.6 × 99 cm) of Sepharose CL-2B by upward elution with degassed sodium acetate buffer (pH 4.0; ionic strength, 0.1) at 18 mL/h at room temperature. Samples (4 mg) of pectin were applied to the column and 4-mL fractions were collected. The contents of galacturonic acid and total neutral sugars were measured as described above, and that of feruloyl groups in each fraction from the absorbance at 375 nm, the pH being adjusted at 10 with M NaOH. The results are expressed as a function of $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e, V_o, and V_t are the elution volume of the fraction, the void volume (77 mL, as determined with apple pectin), and the total volume (184 mL, as determined with galacturonic acid), respectively.

RESULTS

Effect of some oxidising agents. — The addition of potassium periodate, potassium permanganate, sodium chlorite, potassium ferricyanide, and hydrogen peroxide severally to a 0.44% solution of sugar-beet pectin caused a continuous decrease of the reduced viscosity. Data obtained after reaction for 400 min are reported in Table II. A marked decrease in viscosity was caused by permanganate ions. The use of higher concentrations of these oxidants caused a faster decrease in viscosity and there was no effect at lower concentrations. Only the agents hydrogen peroxide–peroxidase and ammonium peroxysulfate ("persulfate") increased the viscosity of the mixture. The former increased instantaneously the reduced viscosity of sugar-beet pectin by 40% to a value which decreased only slightly with time (+36 and +34%, after 400 and 950 min, respectively), whereas the latter caused a continuous increase of viscosity (+63% after 400 min). Ammonium persulfate induced a regular decrease in the viscosity of apple pectin (−25 and −53% after 400 and 950 min, respectively).

Evidence for modification of feruloyl groups during the action of persulfate.

TABLE II

ACTION OF SOME OXIDISING AGENTS ON SUGAR-BEET PULP PECTIN AND APPLE PECTIN AT 25° AS MONITORED BY VISCOMETRY

	Oxidant	*Concentration (mM)*	*Changes in reduced viscosity (%) after 400 min*
Sugar-beet pectin (0.44%)	KIO_3	10	−1
	$KMnO_4$	1	−13
	$NaClO_2$	10	−14
	$K_3Fe(CN)_6$	10	−5
	H_2O_2	0.1	−6
	H_2O_2 + peroxidase	[a]	+36
	$(NH_4)_2S_2O_8$	10	+63
Apple pectin (0.2%)	$(NH_4)_2S_2O_8$	10	−25

[a] 0.1mM H_2O_2; 1.3 mg of peroxidase/L.

— Variations of the reduced viscosity and of the contents of galacturonic acids, total neutral sugars, and feruloyl groups with time when a 0.44% solution of pectin was treated with 0.01M ammonium persulfate at 25° are shown in Fig. 1. The contents of galacturonic acid and total neutral sugars were not changed. In contrast, marked changes in reduced viscosity and in the content of feruloyl groups occurred simultaneously after an induction period of 60–90 min. The content of feruloyl groups decreased continuously, whereas the reduced viscosity reached a maximum

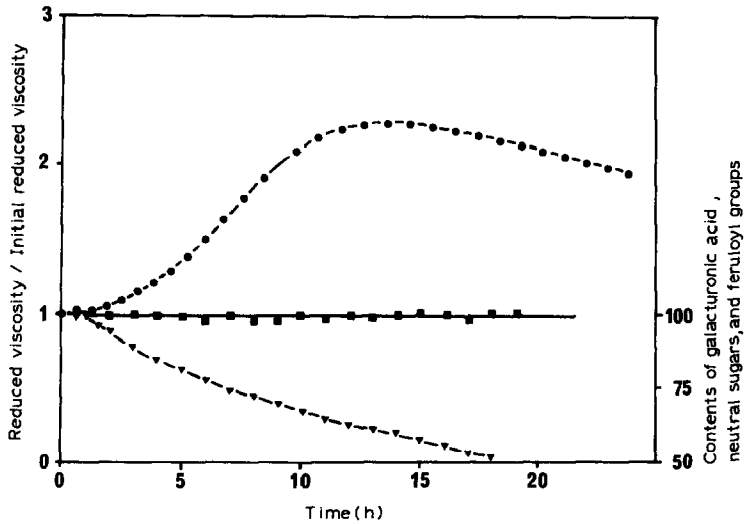

Fig. 1. Changes with time of the ratio of reduced viscosity to initial reduced viscosity (●), and of the contents of galacturonic acids and total neutral sugars (■) and feruloyl groups (▼) expressed as a % of the initial values of a 0.44% solution of pectin in 0.01M ammonium persulfate at 25°.

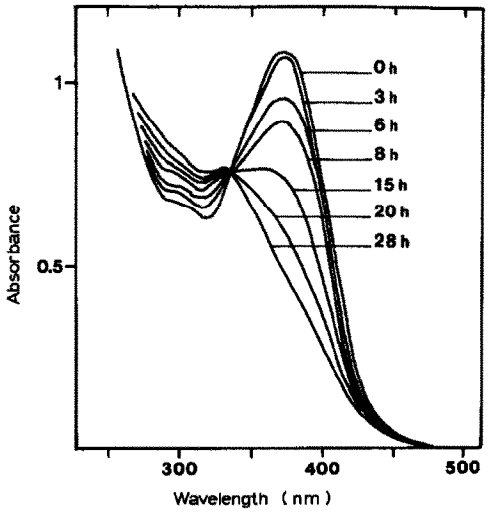

Fig. 2. Changes with time of the u.v. spectra of a 0.44% solution of pectin in 0.01M ammonium persulfate at 25°; the solution (1 mL) was mixed with 0.2M glycine–sodium hydroxide buffer (pH 10, 3 mL) and the spectra were recorded immmediately.

value of 456 mL/g (+130%) after 780 min and thereafter decreased slowly. Fig. 2 illustrates the change in the absorption spectrum of the pectin under alkaline conditions as a function of time. Initially, a peak at 375 nm was observed corresponding to feruloyl groups[21]. The absorbance at 375 nm decreased with increased reaction time and all the curves passed through an isobestic point at 330 nm.

The course of the reaction was also followed by gel-permeation chromatography on Sepharose CL-2B (Fig. 3). The native pectin was characterised by a major peak for galacturonic acid with K_{av} 0.85 with a shoulder for lower K_{av} values, and by two peaks at K_{av} 0.85 and 0.6 where materials containing both neutral sugars and feruloyl groups were eluted. As the reaction proceeded, an increasing amount of material was eluted in the void volume: 4, 30, and 39% of the total galacturonic acids and 8, 43, and 54% of the neutral sugars, initially and after 9 and 15 h of reaction, respectively. The feruloyl groups also accumulated in this peak. At K_{av} 0.85, a peak was left from which feruloylated material had disappeared.

Influence of pectin concentration. — The extent of the reaction with 0.01M ammonium persulfate at 25° was monitored by viscometry (Fig. 4). When the concentration of pectin was 0.2%, only a slight increase in viscosity (+55% after 790 min) was observed after an induction period of ~150 min. On increasing the concentration, the induction period decreased, whereas the rate of the increase in viscosity as well as the maximum value of the viscosity increased. For example, the maximum value of the reduced viscosity of a 0.6% solution of pectin was 1245 mL/g after 1230 min; this value decreased slowly thereafter. At higher concentrations of pectin, gels were formed, namely, after 783, 465, and 410 min with pectin concentrations of 0.8, 1, and 1.2%, respectively.

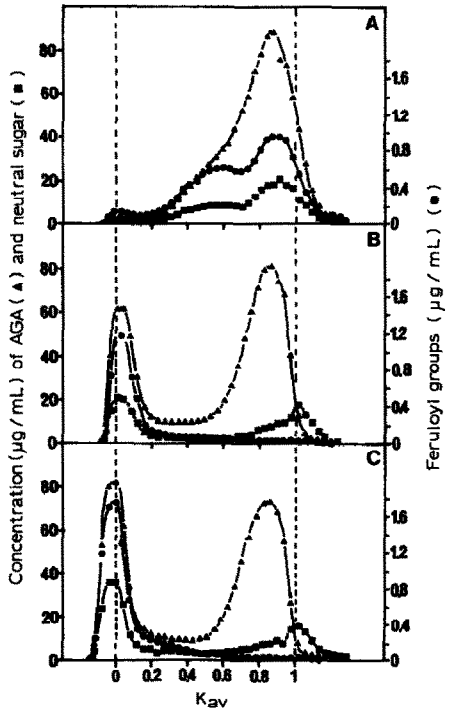

Fig. 3. Elution profiles on Sepharose CL-2B of native pectin (A) and of a 0.44% solution of pectin treated in 0.01M ammonium persulfate at 25° for 3 h (B) and 15 h (C) (AGA = "anhydrogalacturonic acid").

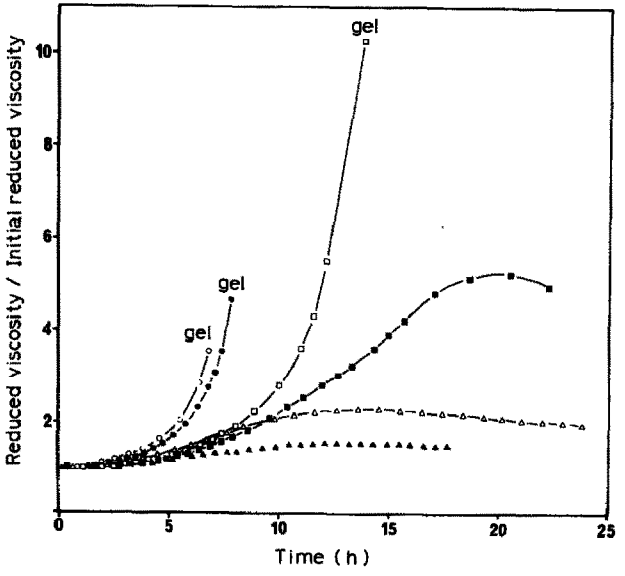

Fig. 4. Changes with time of the ratio of reduced viscosity to initial reduced viscosity of a solution of pectin at various concentrations (▲, 0.25; △, 0.44; ■, 0.6; □, 0.8; ●, 1; ○, 1.2%) in 0.01M ammonium persulfate at 25°.

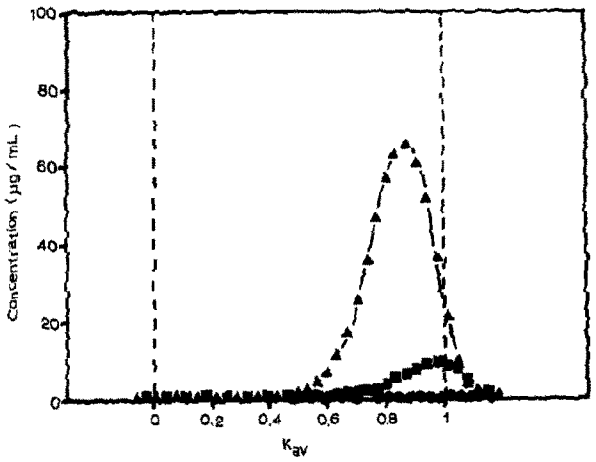

Fig. 5. Elution pattern on Sepharose CL-2B of the water-soluble products obtained after the action of 0.01M ammonium persulfate on a 1.2% solution of pectin at 25°: galacturonic acid, ▲; neutral sugars, ■; feruloyl groups, ●.

These gels contained water-soluble material (53.4% of galacturonic acid) representing 33% of the initial weight of the pectin, which was easily eliminated by aqueous extraction. Analysis of the initial pectin and of the gelled pectin (Table I) showed that the carbohydrate composition of these two products was similar, with a preponderance of galactose (11–12%) and with only ~2% of arabinose probably due to the conditions used for the extraction of the pectin.

When chromatographed on Sepharose CL-2B (Fig. 5), the water-soluble material was characterised by a single peak of galacturonic acid and neutral sugars which was eluted at K_{av} 0.85 and contained no feruloyl groups.

Influence of the persulfate concentration. — The effect in the range tested (1–40mM) on a 0.44% solution of sugar-beet pectin was tested at 25° by viscometry. At 1mM, no increase of the viscosity was observed, whereas at 3mM there was a slight increase (+20%) after 2640 min. When the concentration of persulfate was increased, the induction period was shortened and the rate of increase of viscosity was greater (Fig. 6). The maximum values of the reduced viscosity were obtained after 1690, 920, 520, 357, and 114 min for persulfate concentrations of 5, 10, 15, 20, and 40mM, respectively, but these values were lowered by an increase in the concentration of persulfate and the reduced viscosities decreased more rapidly above these maxima. For example, the final viscosity obtained when the pectin solution was treated with 40mM ammonium persulfate reached a value close to the initial one, and chromatography on Sepharose CL-2B showed that the amount of the material which emerged from this column in the void volume slightly decreased with increasing time of reaction: 38.6, 35.1, and 30% of the total galacturonic acids

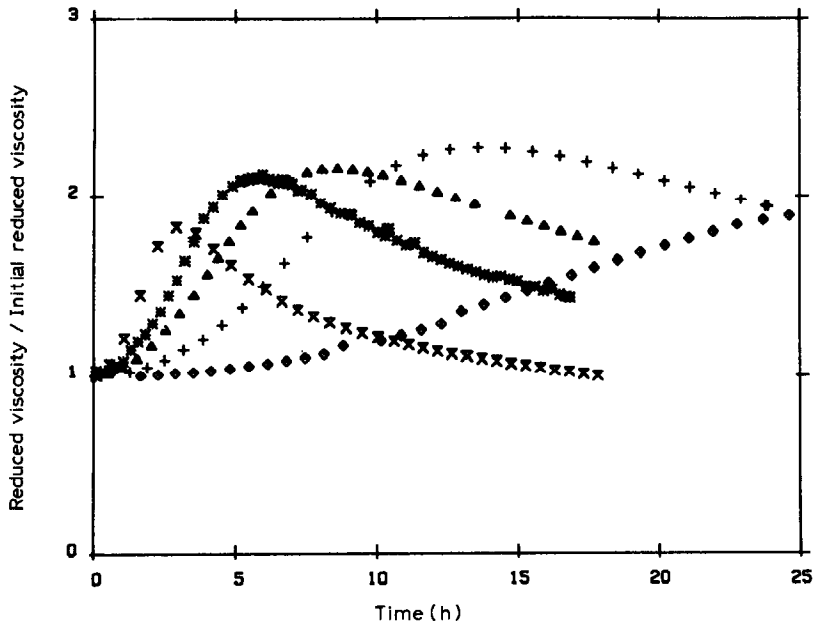

Fig. 6. Changes with time of the ratio of reduced viscosity to initial reduced viscosity of a 0.44% solution of pectin at 25° in ammonium persulfate (⧖, 40; ✱, 20; △, 15; +, 10; ◇, 5mM).

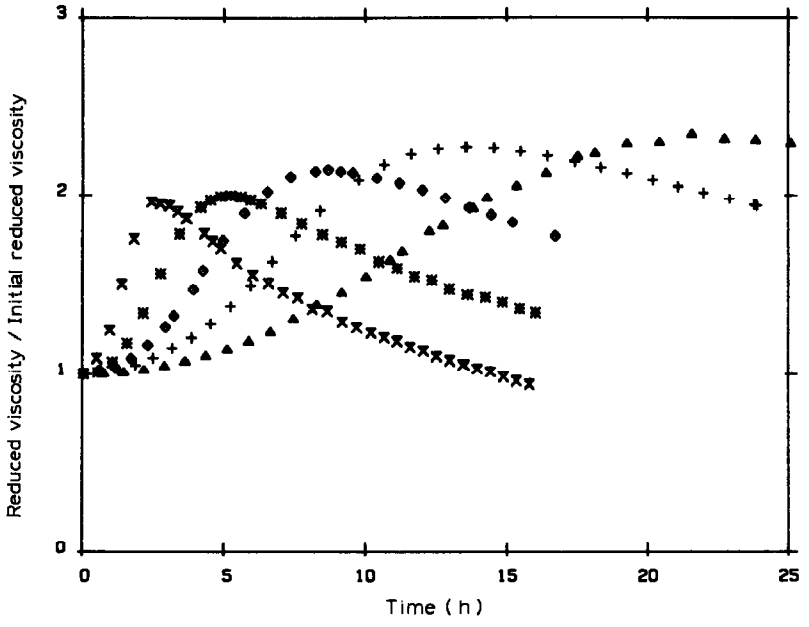

Fig. 7. Changes with time of the ratio of reduced viscosity to initial reduced viscosity of a 0.44% solution of pectin in 0.01M ammonium persulfate at various temperatures (⧖, 40°; ✱, 35°; ◇, 30°; +, 25°; △, 20°).

and 46.5, 44.1, and 43.7% of the total neutral sugars after 3, 18, and 28 h, respectively.

Influence of temperature. — The effect in the range 20–40° was studied by measuring the viscosity of a 0.44% solution of pectin in 0.01M ammonium persulfate. The curves (Fig. 7) are similar to those obtained on varying the concentration of persulfate. Increase in the temperature decreased the induction period and increased the rate of the reaction although the maximum values of reduced viscosity were decreased. These values (504, 456, 397, and 352 mL/g) were obtained after 1500, 900, 540, 300, and 180 min for reactions carried out at 20, 25, 30, 35, and 40°, respectively.

Influence of some additives. — The effect of 0.1M sodium acetate, 10% of 1-propanol, 0.05M disodium hydrogenphosphate, 0.05M sodium dihydrogenphosphate, and 0.05M trisodium citrate on the viscosity of a 0.44% solution of pectin indicated complete inhibition of the reaction (up to 24 h). In contrast, the addition of sodium chloride or sodium sulfate depressed the reaction only slightly (Fig. 8).

DISCUSSION

Sugar-beet pectins can be cross-linked by the action of hydrogen peroxide–peroxidase[8]. Ammonium persulfate can also cause the coupling reaction. The other

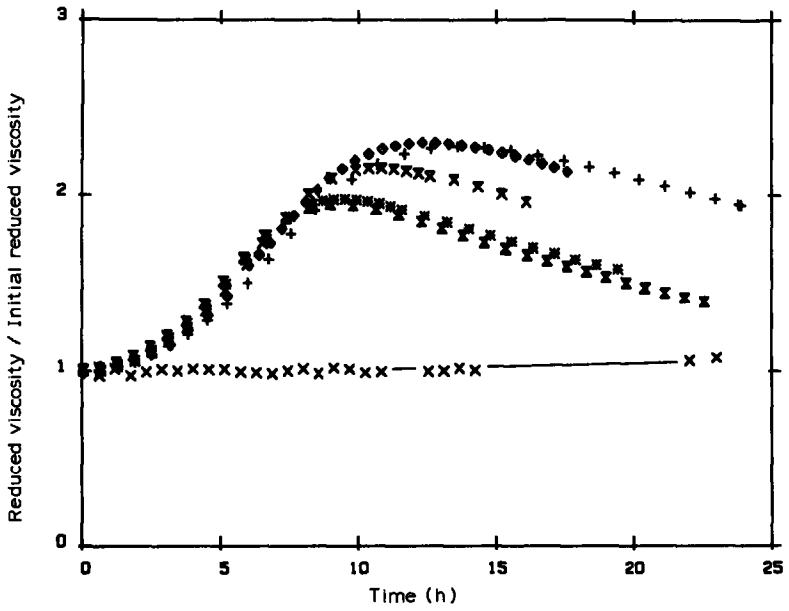

Fig. 8. Influence of the addition of 0.01M NaCl (◇), 0.05M NaCl (⧖), 0.1M NaCl (✱), 0.05M Na_2SO_4 (⧗), and 0.05M NaH_2PO_4 (×) on the changes with time of the ratio of reduced viscosity to initial reduced viscosity of a 0.44% solution of pectin in 0.01M ammonium persulfate at 25° (+, no salt added).

oxidising agents tested (Table II) induced some depolymerisation of the pectic molecules in accord with published data[22,23]. This result disagrees with that of Neukom and Markwalder[13] who reported that no specific oxidants were required for the gelation of feruloylated wheat pentosans, but accords with that of Hoseney and Faubion[11] who found that only hydrogen peroxide–peroxidase, formamidine disulfide, and ammonium persulfate were effective, although they recorded only limited increases in viscosity.

These specific agents are known to generate free radicals[11,24]. The persulfate ion decomposes in aqueous solution to give sulfate radicals and is used in the presence of different reducing agents or alone[24,25] for aqueous polymerisation of acrylamide, methacrylamide, acrylonitrile, or methylmethacrylamide. That the reaction of sugar-beet pectin with persulfate ions involved the formation of free radicals was confirmed by the inhibition effected by the addition of 1-propanol, acetate, citrate, or phosphate ions, which scavenge free radicals[24–26].

The carbohydrate moiety of the pectins was not modified during the reaction with persulfate ions, which ruled out the possibility of cross-linking of modified and unmodified uronic acid or neutral sugar residues in separate chains as described by Fransson[27] for iduronic acid residues in dermatan sulfate. After an induction period, the feruloyl content of the sugar-beet pectins decreased and the viscosity increased. The behaviour of the products on Sepharose CL-2B and the formation of gels were suggestive of random cross-linking. Further, the gelled pectins contained some water-soluble material which was feruloylated, indicating that not all of the neutral-sugar side chains were esterified by ferulic acid. Pectin molecules with and without feruloyl groups cannot be distinguished by their neutral sugar composition (Table I), which is characterised by the preponderance of galactose with only minor amounts of arabinose. The position of the feruloyl groups cannot be deduced from these data, but Fry[9] demonstrated that non-reducing terminal arabinosyl and galactosyl groups are feruloylated.

All of these results indicated that feruloyl groups were involved in the reaction, but it is not possible to conclude that the cross-linking resulted in dimerisation of feruloyl residues belonging to different pectic molecules. The covalent binding of associated proteins and feruloyl groups of pectic chains, as formulated by Hoseney and Faubion[11] or by Neukom and Markwalder[13] for the gelation of wheat pentosans, cannot be ruled out.

The influence of persulfate and pectin concentration, temperature, and the presence of additives was studied by viscometry. However, the viscosity depends not only on the concentration of the polymer but also on the molecular weight and shape, pH, and ionic strength, which can vary with the time of reaction. Solutions of sugar-beet pectins behaved as Newtonian fluids if the concentration of the polymer was $<0.75\%$, whereas they were slightly shear-thinning at higher concentrations (data not shown). Persulfate ions decompose in aqueous solutions, probably leading to changes in ionic strength and pH and consequently in viscosity. Therefore, the values of viscosity reported here are only apparent and an increase

revealed only that the reaction was progressing. Furthermore, chromatography on Sepharose CL-2B showed, in accord with the viscosity results, that some depolymerisation or changes in the stiffness of the molecules[28] occurred when sugar-beet pectin was treated with a high concentration of ammonium persulfate. Nevertheless, the viscometry method can be used to follow the cross-linking of the sugar-beet pectin and, in order to determine the optimal conditions, account must be taken of the production of free radicals by decomposition of persulfate ions as well as the chemical stability of the pectic molecules. The reaction was strongly dependent on the concentration of persulfate and pectin and on temperature, leading to different molecular weights or shapes, as judged by viscometry. The best conditions for obtaining either pectins having increased molecular weight or gels appeared to involve 0.01M ammonium persulfate at 25°.

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