ELSEVIER

PII: S0144-8617(97)00060-X

Carbohydrate Polymers 34 (1997) 67–71

0144-8617/97/$17.00 + .00

Some properties of *ruredzo* (*Dicerocaryum zanguebarium*) mucilage crosslinked with epichlorohydrin

Mudadi A. N. Benhura and Isabella Mavhudzi-Nyambayo

Department of Biochemistry, University of Zimbabwe, P.O. Box MP 167, Mount Pleasant, Harare, Zimbabwe

(Received 26 September 1996; revised version received 16 February 1997; accepted 14 March 1997)

The yield of crosslinked mucilage (CLM) prepared from *ruredzo* mucilage, using epichlorohydrin as the crosslinking agent, decreased by about 10% when the concentration of ethanol was reduced from 96 to 40%. CLM prepared at low concentration of mucilage was more swellable than CLM prepared from mucilage at higher concentrations. The CLM was insensitive to the presence of NaCl but the bed volume was reduced in the presence of ethanol. Bed volume was lower at low pH than at high pH. When CLM was incubated with Pectinex, practically no change was observed in bed volume but arabinose and mannose were detected in the liquid overlying the treated CLM. Galactose and arabinose, which are the main components of *ruredzo* mucilage, were not released during incubation of the crosslinked mucilage with Pectinex. Copyright © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

The plant *ruredzo* (*Dicerocaryum zanguebarium*) grows widely in the sandy soils of the veld in southern Africa. The mucilage from *ruredzo* is a pectin-like polymer whose presence in leaves was probably the basis of traditional uses of the plant. The leaves were used not only as food but also for treating measles and facilitating births in domestic animals and humans (Benhura & Marume, 1993). Native *ruredzo* mucilage has been reported to contain galactose, xylose, arabinose and mannose in the ratio 21:19:12:1. We have been trying to find applications for the mucilage that is isolated from *ruredzo*. Epichlorohydrin (ECH) has been used for crosslinking citrus pectin in alkaline conditions and in the presence of ethanol. In the crosslinking procedure, dimethylsulphoxide (DMSO) may be used as a solvent instead of ethanol.

In this study, we wanted to evaluate some properties of the crosslinked mucilage that is prepared from the mucilage from *ruredzo*. The operation of a crosslinked polysaccharide as, for example, an adsorbent matrix is likely to be influenced by a variety of environmental factors. We wanted to investigate the effect of some of these factors on the behaviour of crosslinked *ruredzo* mucilage.

EXPERIMENTAL

Preparation of mucilage

Mucilage was extracted from ground dried leaves with boiling water and purified with copper acetate as described earlier (Benhura & Marume, 1993).

Crosslinking of *ruredzo* mucilage with epichlorohydrin

A stock solution of mucilage 2% (w/v) was prepared by suspending 2 g of mucilage in distilled water, leaving overnight at 4°C and, after hydration, making up the volume to 100 ml with distilled water. The final concentration of the solution of mucilage was determined by drying aliquots in an oven at 100°C. Purified mucilage, at concentrations up to 2%, was crosslinked with ECH using the homogeneous mode (Benhura & Mavhudzi, 1996)

The yield of the dry CLM product was determined

on the basis of the mass of the dry mucilage that was used in the crosslinking reaction (Kunaik *et al.*, 1972; Hatanaka *et al.*, 1990).

Characterisation of crosslinked mucilage

Measurement of moisture content
The CLM, consecutively dried in ethanol, acetone and petroleum ether, was dried overnight at 100°C in a pre-heated oven.

Estimation of free carboxyl groups
CLM was converted to the H^+ form by incubating overnight in 1 M HCl (Vogel, 1978). The CLM was washed with water until the washings were neutral to litmus paper. To 1 g of treated CLM was added degassed deionised water (100 ml) and NaCl (1 g), and the mixture was titrated against standardised 0.1 M NaOH using phenolphthalein as the indicator. A titre of 1 ml was taken to be equivalent to 0.1 meq of free carboxyl or 17.6 mg of anhydrogalacturonic acid (Hatanaka *et al.*, 1990).

Evaluation of bed volume of CLM
CLM (1 g) was suspended in 20 ml distilled water in a 25 ml measuring cylinder. The level of gel was read after 6 h of incubation at room temperature (Kunaik *et al.*, 1972; Kohn *et al.*, 1976; Rombouts *et al.*, 1979; Inoue *et al.*, 1984; Hatanaka *et al.*, 1990; Moe *et al.*, 1993).

Effect of concentration of ethanol on bed volume
Dried CLM (1 g) was suspended in 20 ml distilled water in a 25 ml measuring cylinder. The suspension was allowed to stand overnight at 4°C. The distilled water was decanted and the swollen gel resuspended in up to 98% ethanol (20 ml). The level of gel was recorded within 6 to 72 h of incubation at 4°C (Moe *et al.*, 1993).

Effect of ionic strength on bed volume
CLM was pre-swollen by suspending in distilled water and the gels resuspended in up to 1 M NaCl (20 ml) in a 25 ml measuring cylinder. The level of gel was recorded within 6 to 72 h of incubation at 4°C (Moe *et al.*, 1993).

Effect of pH on bed volume
CLM was swollen in distilled water and excess water removed by decanting. The gel was resuspended in 0.1 M citric–0.2 M phosphate buffer (20 ml) of pH 2 to 7 in a 25 ml measuring cylinder. The level of gel was recorded within 6 to 72 h of incubation at 4°C (Moe *et al.*, 1993).

Estimation of ion exchange capacity of CLM
The CLM was converted to the H^+ form by incubating overnight in 1 M HCl (Vogel, 1978). The CLM was washed with water until a neutral pH was detected in the washings using litmus paper. To 1 g of treated CLM was added degassed deionised water (100 ml) and the mixture was titrated against standardised 0.1 M NaOH using phenolphthalein as the indicator. The ion-exchange capacity was obtained as TA/W meq/g where T is the volume of 0.1 M NaOH, A is the molarity of 0.1 M NaOH and W is the mass of resin taken (Vogel, 1978).

Biodegradation of crosslinked mucilage
CLM (1 g) was pre-swollen in distilled water and the pH of the mixture adjusted to 4. The gel was suspended in Pectinex UTL-SP (6 ml, pH 4) and 0.2 M acetate buffer (14 ml, pH 4) in a 25 ml measuring cylinder. The reaction mixtures were incubated at 4°C for up to 15 days. Aliquots of 1.5 ml were sampled during incubation and the reaction stopped by heating in a boiling water bath for 10 min. The reducing groups generated during incubation were determined using the dinitrosalicylic acid (DNSA) method. The level of the gel was recorded on every sampling done. The reaction mixture was centrifuged after incubation for 15 days and the supernatant divided into two portions. The first portion was dried in an oven pre-heated to 100°C to dryness and the second portion was dried in a Christ Alpha 2-4 model freeze drier. The released monosaccharides were detected using thin layer chromatography.

Determination of reducing sugars
To samples, standards and controls (100 µl), 1 ml of the DNSA reagent was added and the mixture heated for 10 min in a boiling water bath. The mixtures were cooled rapidly to room temperature under running tap water. The absorbance was measured at 570 nm using a Shimadzu UV 160 A spectrophotometer (Chaplin, 1986). The calibration curve was prepared using galactose as the standard reducing sugar.

Detection of reducing sugars by thin layer chromatography
The samples were reconstituted in 1 ml of distilled water. The constituent monosaccharides were determined by spotting the degraded samples on commercial silica gel 150 A KS 20×20 cm plates. Standards were prepared by dissolving 0.05 g in 5 ml of distilled water to make 1% solutions. Sigma micropipettes were used to spot 4 µl of standards and up to 25 µl of samples. The plates were left to dry at room temperature.

Ethyl acetate/pyridine/water (10:3.5:2.5) was used to develop the plates for 2 h, after which the spots were detected using diphenylamine/aniline/orthophosphoric acid reagent (Chaplin, 1986).

Table 1. Some properties of the crosslinked mucilage prepared from different concentrations of solutions of *ruredzo* mucilage at concentrations up to 2%. The uncertainties shown are standard deviations for at least three replicates

Concentration of mucilage (%)	0.25	0.5	1.0	2.0
Yield (%)	92.7±1.2	90.1±0.5	95.0±2.5	95.9±2.0
Moisture (%)	11.1±1.9	11.5±0.5	12±2	12.4±1.5
Free carboxyl (meq/g)	1.00±0.01	1.06±0.06	1.03±0.05	0.99±0.01
Ion-exchange capacity (meq/g)	1.1±0.1	1.14±0.01	1.17±0.01	1.24±0.01

RESULTS AND DISCUSSION

It can be seen in Table 1 that the properties of CLM that was prepared using mucilage of different concentrations are similar. Higher concentrations of mucilage resulted in slightly higher yield of CLM that was slightly more difficult to dry. The properties of CLM prepared from *ruredzo* under different concentration are similar to those that were observed for the crosslinked pectate from citrus peels (Hatanaka *et al.*, 1990). The ion-exchange capacity of *ruredzo* CLM was higher than that reported for carboxymethyl cellulose in the fibrous microgranular form (Khym, 1974). When 40% ethanol was used as the solvent instead of 96% ethanol, the yield of CLM dropped from 93 to 85% and the bed voloume was halved as shown in Table 3. The CLM prepared using ethanol as a solvent is highly hygroscopic.

The CLM prepared from 0.25% solution of mucilage was considerably more swellable than CLM prepared from mucilage at higher concentrations, as shown in Fig. 1. It appears that as the extent of crosslinkage between chains increased, the CLM became more compact.

The swelling of a matrix in water results from the tendency of the functional groups to become hydrated. The extent of swelling depends on the number of hydrophilic functional groups attached to the polymer matrix and is inversely proportional to the degree of crosslinking. The degree of crosslinking would be expected to be a more important factor in determining to what extent a resin is free to swell or to shrink. With a low degree of crosslinkage, the network is more easily stretched and the swelling is large.

It can also be seen in Fig. 1 that the bed volume of CLM decreased when suspensions of CLM were left to stand, with the CLM prepared from the 2% solution of mucilage showing the smallest variation in bed volume over time. Thus the gel formed at low concentrations of mucilage was not only more swellable but was also more compressible than CLM formed at high concentrations of mucilage.

The effect of ethanol on *ruredzo* CLM is shown in Fig. 2, from which it can be seen that the effect of ethanol was more marked with CLM prepared from lower concentrations of mucilage solution. As shown in Fig. 3, increasing the concentration of NaCl in the suspending medium resulted in only a slight decrease of the bed volume of CLM. In Fig. 4 it can be seen that the bed volume of *ruredzo* CLM was smaller at low pH than at high pH.

In general, the degree of swelling of CLM prepared using the 2% solution was less affected by ethanol, sodium chloride, pH and time of incubation at 4°C than mucilage prepared at a lower concentration of mucilage. The biggest change in bed volume was

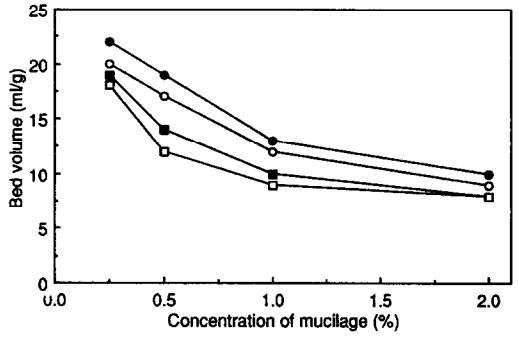

Fig. 1. The bed volume of CLM prepared from different concentrations of solution of mucilage. CLM (1 g) was suspended in 20 ml of distilled water for 6 h at room temperature and the bed volume recorded after 6 h (–•–), 24 h (–○–), 48 h (–■–) and 72 h (–□–) at 4°C.

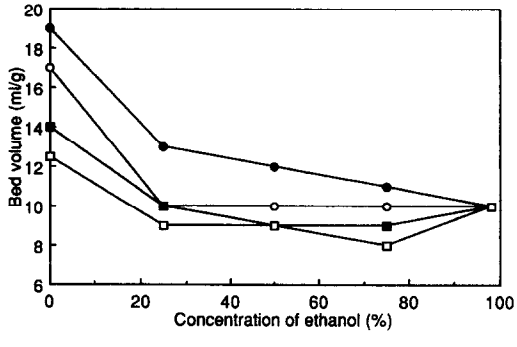

Fig. 2. Effect of ethanol on bed volume of CLM. CLM prepared from solutions of mucilage at concentrations of 0.25% (–•–) 0.5% (–○–), 1% (–■–) and 2% (–□–) was suspended in water containing up to 96% ethanol and the bed volume recorded after 6 h of incubation at 4°C.

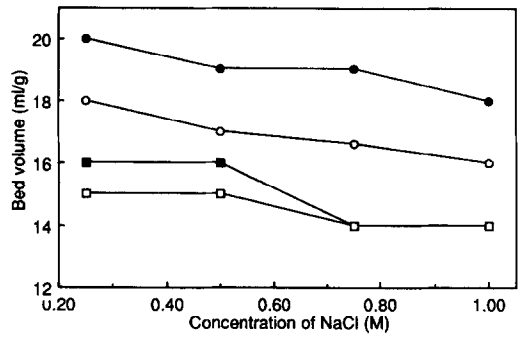

Fig. 3. Effect of NaCl on bed volume of CLM. CLM prepared from solutions of mucilage at concentrations of 0.25% (-•-), 0.5% (-○-), 1% (-■-) and 2% (-□-) was suspended in 20 ml of up to 1 M NaCl and the bed volume recorded after 6 h of incubation at 4°C.

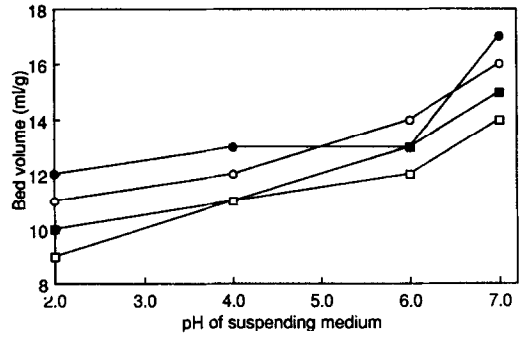

Fig. 4. Effect of pH on bed volume of CLM. CLM prepared from 0.25% of solution of mucilage was suspended in buffers (20 ml) at pH up to 7 and the bed volume recorded after 6 h (-•-), 24 h (-○-), 48 h (-■-) and 72 h (-□-) at 4°C.

observed when the suspending medium was changed from distilled water to one of the above environmental conditions.

The CLM prepared from solutions of mucilage with concentration lower than 2% had their bed volume affected by environmental factors such as ionic strength, pH and the presence of solvents such as ethanol. These factors would, therefore, need to be controlled in any practical usage of the crosslinked polymer prepared from solutions of low concentrations of mucilage.

The release of reducing groups by CLM that had been incubated with Pectinex is shown in Table 2. The amount of reducing groups released during incubation of CLM with the pectin-degrading enzyme increased. However, only a small change in the bed volume of the CLM was observed during the 15 day incubation period. Mannose and arabinose were detected in the freeze-dried liquid that was separated from the CLM that remained undegraded after treatment with Pectinex. The main components of *ruredzo* mucilage, galactose and arabinose were not released during incubation with Pectinex. It appears that hemicellulases in the commercial enzyme preparation degraded accessible side chains that contained arabinose and mannose with the main galactose chain remaining unaffected. The fact that galactose residues were not released by the action of Pectinex could indicate that galactose residues were in the main chain where they occur in esterified uronic acid forms. Sections of the polymer containing esterified galacturonic acid residues could be resistant to the action of polygalacturonases.

Table 2. Release of reducing groups when suspensions of *ruredzo* CLM, prepared using 0.5% solution of mucilage, was incubated with Pectinex at 4°C. Reducing groups were determined by the DNSA method. The uncertainties shown are standard deviations for duplicate analysis

Time of incubation	Bed volume (ml/g)		Reducing groups (μg/ml)
	Blank	Sample	
0 h	11.0±0.5	8.5±0.5	3
4 h	8.5±0.5	8.0±0.5	7
8 h	8.0±0.5	7.5±0.5	7
24 h	7.5±0.5	7.5±0.5	8
4 days	7.0±0.5	7.0±0.5	9
15 days	7.0±0.5	7.0±0.5	31

Table 3. Comparison of properties of *ruredzo* CLM prepared from 0.5% solution of mucilage using ethanol at different concentrations, as the solvent. The uncertainties shown are standard deviations for at least three replicates

Property	Concentration of Ethanol (%)	
	40	96
Yield (%)	85.3±1.0	93.4±1.0
Moisture (%)	11.0±0.8	13.0±1.0
Bed volume (ml/g)	15.0±0.5	31.0±0.5
Free Carboxyl (meq/g)	0.92±0.01	1.03±0.06

ACKNOWLEDGEMENTS

This study was generously supported by grants from the International Foundation for Science (IFS), the Research Board of the University of Zimbabwe and the Swedish Agency for Research Cooperation with Developing Countries (SAREC).

REFERENCES

Benhura, M.A.N. and Marume, M. (1993) The mucilaginous polysaccharide material isolated from *ruredzo* (*Dicerocaryum zanguebarium*). *Food Chem.* **46,** 7–11.

Benhura, M.A.N. and Mavhudzi, I. (1996) Use of crosslinked mucilage prepared from *ruredzo* (*Dicerocaryum zanguebarium*) in the purification of polygalacturonase extracted from tomato. *Food Chem.* **56**(4), 433–437.

Chaplin, M.F. (1986) Monosaccharides, in *Carbohydrate Analysis—A Practical Approach*, ed. M.F. Chaplin and J.F. Kennedy, pp. 1–36. IRL Press, Oxford.

Hatanaka, C., Sakamoto, K. and Wada, Y. (1990) Preparation of a water-soluble hard gel, crosslinked cell walls, from citrus peels. *Agric. Biol. Chem.* **54**(12), 3347–3348.

Inoue, S., Nagamatsu, Y. and Hatanaka, C. (1984) Preparation of crosslinked pectate and its application to the purification of endopolygalacturonase of *Kluyveromyces fragilis*. *Agric. Biol. Chem.* **48**(3), 633–640.

Khym, J.X. (1974) *Analytical ion exchange procedures in chemistry and biology. Theory, Equipment, Techniques*, 70 pp. Prentice-Hall Inc., Englewood, New Jersey.

Kohn, R., Rexová-Benkova, L'., Luknar, O. and Kuniak, L. (1976) Crosslinked pectic acid. The effect of crosslinking on cation exchange, binding of endoploygalacturonase and biodegradability. *Coll. Czecho. Chem. Commun.* **41,** 1878–1893.

Kunaik, L., Marchessault, R.H. and Que, M. (1972) Study of the crosslinking reaction between epichlorohydrin and starch. *Starch (Stärke)* **24,** 110–116.

Moe, S.T., Skjak-Braek, G., Elgstaeter, A. and Smidsrød, O. (1993) Swelling of covalently crosslinked alginate gels: influence of ionic solutes and non-polar solvents. *Macromolecules* **26**(14), 3589–3597.

Rombouts, F.M., Wissenburg, A.K. and Pilnik, W. (1979) Chromatographic separation of orange pectinesterase isoenzymes on pectates with different degrees of crosslinking. *J. Chrom.* **168,** 151–161.

Vogel, A.I. (1978) *A Textbook of Quantitative Inorganic Analysis Including Elementary Instrumental Analysis*. 4th edn, pp. 165–181, London. Longman.