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Preliminary communication

Improvements in the methanolysis of pectins by enzymic prehydrolysis

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Determination of the constituents of polysaccharides generally involves hydrolysis with sulfuric[1] or trifluoroacetic acid[2]. The various stabilities of the free monosaccharides necessitate different optimum conditions of hydrolysis. Methanolysis causes less destruction than does aqueous acid[3], but the release of uronic acids from such anionic polymers as pectins[4], xanthan[4], or alginates[5] is not complete. In order to improve the methanolysis of pectins, enzymic prehydrolysis[6] was carried out. The methyl glycosides released were analyzed by h.p.l.c.[7,8], which is less tedious than g.l.c. of the trimethylsily ethers[9–11]. The results were compared with those obtained by methanolysis with (*1*) M HCl in methanol, and (*2*) H_2SO_4 in methanol (with pretreatment[12] for 3 h with aqueous 72% H_2SO_4), by colorimetric determination[13] of the content of galacturonic acid, as well as by g.l.c. of the alditol acetate derivatives[14] of the neutral sugars released by hydrolysis with sulfuric and trifluoroacetic acid.

The sugar beet pectin (57.8% of galacturonic acid[13]) and the citrus pectin (degree of methylation, 72%; 79.2% of galacturonic acid) were obtained from Københavns Pectinfabrik (Copenhagen, Denmark); the apple pectin (degree of methylation, 28%; 76.8% of galacturonic acid) was from Unipectine (Redon, France). The polysaccharides were purified by precipitation from their aqueous 2% solutions with ethanol (4 vol.) or by precipitation with Cu^{2+} ions (apple pectin only). The sugar beet pectin was dehydrated by solvent exchange (ethanol, acetone, ether) and ground (particle size <0.5 mm). The citrus and apple pectins were freeze-dried. All the samples were dried at 40° over P_2O_5, under vacuum for at least 24 h. The enzyme used was a commercial liquid preparation (SP 249) obtained from NOVO (Denmark) and contained[15] mainly the following activities: polygalacturonase (EC 3.2.1.15), pectinesterase (EC 3.1.1.11) pectin lyase (EC 4.2.2.10), α-L-arabinofuranosidase (EC 3.2.1.55), β-D-galactosidase (EC 3.2.1.23),

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arabinanase (EC 3.2.1.99), and galactanase (EC 3.2.1.89). A portion (5 mL) was purified by precipitation with $(NH_4)_2SO_4$ (90% of saturation) in order to eliminate the low-molecular-weight sugars. After centrifugation, the pellet was washed with $(NH_4)_2SO_4$ at 90% saturation (2 × 100 mL) and a solution in 50mM acetate buffer (5 mL, pH 4.5) was dialysed at 4° for 8 h against four changes of ultrapure water (to a conductivity <10 μSiemens/cm), then for 4 h against two changes of 50mM

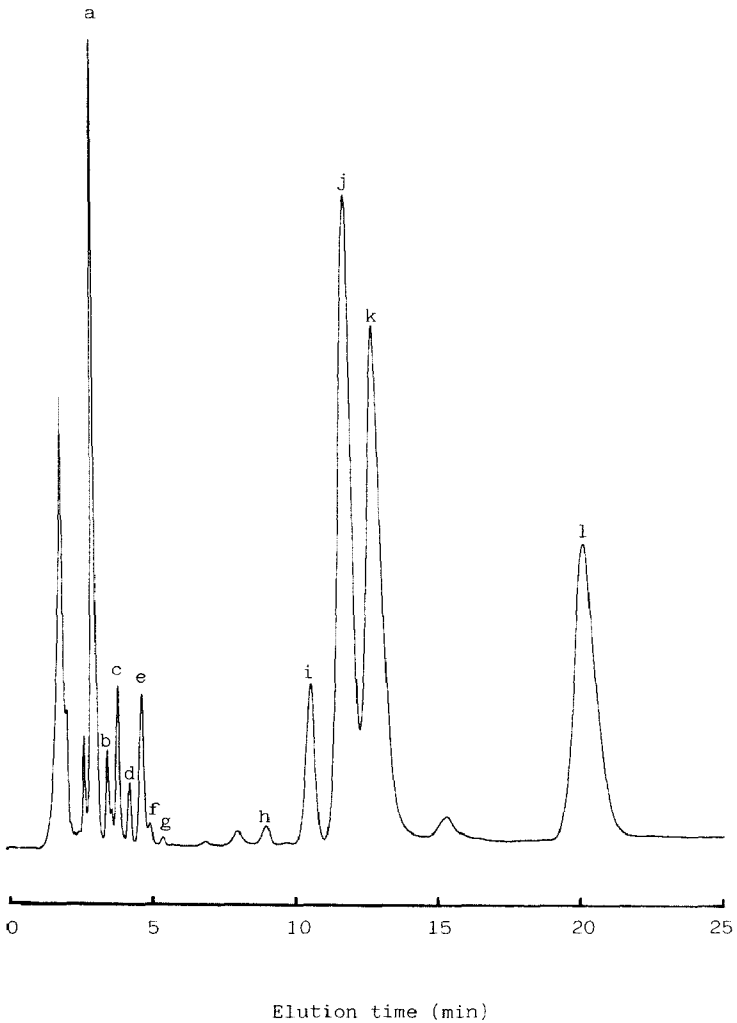

Fig. 1. H.p.l.c. of methanolysis products from sugar beet pectin: a, methyl α- and β-D-galactopyranosides; b, methyl α-D-galactofuranoside and methyl α- and β-D-glucopyranosides; c, methyl β-D-galactofuranoside and methyl α-L-arabinofuranoside; d, methyl β-L-arabinofuranoside; e, methyl α- and β-L-arabinopyranosides; f, methyl β-D-mannopyranoside and methyl α- and β-D-mannofuranosides; g, methyl β-D-xylopyranoside; h,i, methyl L-rhamnosides; j, methyl (methyl α- and β-D-galactofuranosid)-uronates; k, methyl (methyl α- and β-D-galactopyranosid)uronates; l, dimethyl L-tartrate (internal standard).

acetate buffer, and diluted in ultrapure water to a protein concentration[16] of 1 mg/mL.

The improved methanolysis procedure was conducted in a Reacti-Therm Heating/Stirring Module (Pierce and Warriner). The dried pectin (10 mg) was incubated in a 10-mL Reacti-Vial at 45° for 30 min with purified enzyme preparation (400 μL). The hydrolysate was concentrated to dryness with 500 μL of methanol at 45° in a stream of air, using the Reacti-Vap Evaporator. The process was repeated twice more, methanolic M HCl[3] (2 mL) containing dimethyl L-tartrate as internal standard[8] (2 mg/mL) was added, the tube was sealed with a Teflon-lined septum, and methanolysis was then carried out at 85° for 24 h. Following neutralisation by Ag_2CO_3 (120 mg) and centrifugation (5000*g* for 5 min), the supernatant solution was concentrated (40°, under vacuum) and the residue was dissolved in water (2 mL). The methyl glycosides were fractionated on a Merck Superspher C18 cartridge (25 × 0.4 cm) by elution at 1 mL/min with water. The eluate was monitored by differential refractometry. For quantification, the response factors of standard sugars relative to that of the internal standard were calculated from the main glycoside peak, namely, that which was well separated from those of other derivative sugars (a, b, e, g, i, and j + k, for galactose, glucose, arabinose, xylose, rhamnose, and galacturonic acid, respectively; Fig. 1). In the determination of glucose, problems can arise due to the presence of methyl α-D-galactofuranoside and methyl α- and β-D-glucopyranosides in peak b. As the proportions of the areas of the peaks corresponding to the derivatives from the standard sugar (galactose) are constant for concentrations of ≤5 mg/mL, it is possible to determine, from the area of peak a, the area corresponding to methyl α-D-galactofuranoside under peak

TABLE I

NEUTRAL SUGAR AND GALACTURONIC ACID CONTENTS (%) OF PECTINS

Pectin	*Method*	*GalA*	*Rha*	*Fuc*	*Ara*	*Xyl*	*Gal*	*Glc*
Sugar beet	M H_2SO_4, 100°, 2 h		1.9	0.10	3.3	0.15	7.8	0.10
	2M CF_3COOH, 120°, 2 h		4.8	0.07	3.1	0.20	7.3	0.11
	Methanolysis method *1*	38.5	5.1	nd[a]	3.1	0.15	7.2	nd
	Method *2*	42.6	4.0	nd	nd	nd	nd	nd
	Proposed method	57.5	5.1	nd	3.1	0.15	7.5	nd
Apple	M H_2SO_4, 100°, 2 h		1.0	0.05	0.4	1.50	2.8	3.10
	2M CF_3COOH, 120°, 2 h		2.0	0.07	0.5	1.40	2.6	2.60
	Methanolysis method *1*	51.5	2.1	nd	1.0	1.20	2.5	3.50
	Proposed method	74.2	2.2	nd	1.0	1.40	2.6	3.60
Citrus	M H_2SO_4, 100°, 2 h		0.7	0.06	1.1	0.09	2.3	0.09
	2M CF_3COOH, 120°, 2 h		1.2	0.04	1.0	0.16	2.2	0.13
	Methanolysis method *1*	47.4	1.4	nd	1.0	0.08	2.2	nd
	Method *2*	47.2	1.5	nd	nd	nd	nd	nd
	Proposed method	77.1	1.4	nd	1.1	0.20	2.4	nd

[a]Not determined.

b and thence to determine the amount of glucose. For g.l.c., the neutral sugars were converted into the alditol acetates and analyzed at 220° on a fused-silica capillary column (30 m × 0.32 mm i.d.) with DB225 (0.15-μm film) (J & W Scientific). Hydrogen was the carrier gas and *myo*-inositol was the internal standard.

The results are summarized in Table I. Methanolysis with methods *1* and *2* gave low amounts of GalA, regardless of the origin of the pectin. These values were increased significantly after the enzymic prehydrolysis and became close to those determined colorimetrically. It was difficult to determine galactose, glucose, arabinose, and xylose by method *2* because of interfering salts. Method *1* and g.l.c., after acid hydrolysis, gave comparable values for arabinose, xylose, galactose, and glucose. Fucose was not detectable under the conditions used. The content in rhamnose, which is higher with CF_3COOH hydrolysis than with H_2SO_4, was increased slightly by method *1* or by the proposed method. Therefore, it can be concluded that pectins may be analyzed for their main neutral sugars as well as GalA by methanolysis coupled to enzymic prehydrolysis with subsequent h.p.l.c.

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