

The Trisaccharide Fraction of Some Plants Belonging to the Amaryllidaceae

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The naturally occurring isomeric fructosyl-sucroses, 6-kestose, 1-kestose, and neokestose have been separated by thin-layer chromatography. They were further characterized by methylation. The analytical methods described present a relatively simple technique of examining the trisaccharide fraction of fructosan-containing plants.

Bulbs from three plants belonging to the Amaryllidaceae, *Leucojum vernum* L., *Leucojum aestivum* L., and *Galanthus Elwesii*, proved to contain a mixture of 1-kestose (1^F- β -D-fructofuranosyl-sucrose) and neokestose (6^G- β -D-fructofuranosyl-sucrose). Raffinose was not detected.

The naturally occurring fructosyl-sucroses or kestoses have a β -D-fructofuranosyl group linked to one of the primary alcohol functions of sucrose, thus representing the following three structures; 1^F- β -D-fructofuranosyl-sucrose (1-kestose), 6^F- β -D-fructofuranosyl-sucrose (6-kestose) and 6^G- β -D-fructofuranosyl-sucrose (neokestose). They are especially found in fructosan containing plants (reviews¹⁻⁴), a great number of which are of agricultural interest.

The trisaccharide fraction of a fructosan containing plant may be correlated to the structure of the fructosans actually present (levan type, inulin type, or mixed types), or the trisaccharides may be formed by the action of invertases (including sucrose transfructosylases) upon sucrose.^{2,5} Whether the oligofructosides are formed by degradation of fructosans or by transfructosylation, their actual quantity may be of interest in studies concerning the frost resistance of the bulbs.

The isomeric kestoses are, however, difficult to separate and characterize, and a complete analysis of the trisaccharide fraction has been performed only with comparatively few fructosan containing plants.¹⁻⁴ Complete separation by paper chromatography proved to be difficult.⁶ Schlubach and coworkers, however, succeeded in finding a solvent system (isopropanol, 2-butanone, dimethylformamide, water, 50:25:5:20) which separates all three isomers.^{7,8} Electrophoresis⁹ in borate buffer is reported to give a fairly good separation.

The present paper describes the separation of the isomeric kestoses by thin-layer chromatography, Table 1. It is also demonstrated that methylation may be used to confirm the identification of the kestoses. The methylation,

Table 1. Separation of kestoses by thin-layer chromatography on silica gel. Solvent D.

	1-kestose	6-kestose	neokestose
R_F values of test substances	0.39	0.44	0.48
R_F values of Fraction I from the three plants	0.39		0.48

performed on a semi micro-scale, was applied to the unseparated mixture of the two kestoses found.

The bulbs examined in the present work (from *Leucojum vernum* L., *Leucojum aestivum* L., and *Galanthus Elwesii*) all contained a mixture of two of the kestoses; 1^F- β -D-fructofuranosyl-sucrose, and 6^G- β -D-fructofuranosyl-sucrose. Bacon^{1,10} has characterized these two kestoses in *Allium* species by means of chromatography on charcoal and Celite columns. Examining grass species (*Lolium multiflorum* Lam. and *Arrhenatherum elatius* var. *bulbosum*), Bacon found the same two kestoses and also the third isomer, 6^F- β -D-fructofuranosyl-sucrose.

It is hoped that the techniques described in the present paper may contribute to facilitate the further study of the important trisaccharide fraction from fructosan containing plants.

EXPERIMENTAL

The following plants belonging to the *Amaryllidaceae* were examined: *Galanthus Elwesii*, *Leucojum vernum* L., *Leucojum aestivum* L. Bulbs of commercial origin were used.

The following solvent systems (v/v) were used to obtain the chromatographic separations desired: (A) butanol-pyridine-water, 5:3:2. (B) propanol-water, 78:22. (C) propanol-ethyl acetate-water, 7:1:2. (D) butanol saturated with water-methanol, 100:60. (E) benzene-ethanol, 20:3. (F) methyl ethyl ketone saturated with aqueous ammonia (6 %). Spots were revealed by aniline oxalate.

1. *Extraction of the bulbs by methanol.* The dry external scales of the bulbs were removed. The material (from 25-30 bulbs) was chopped, immediately covered with methanol and then left for 4 days at room temperature. The mixture was filtered, and the residue was again extracted by methanol in the same way. The combined filtrates were concentrated under reduced pressure to a brown syrup which by paper-chromatographic analysis proved to contain several amino acids, various oligosaccharides together with minute amounts of fructose — and in some cases (*Leucojum* species) — also of glucose.

The extracted material was dried in an air stream and then finally dried at 40°C.

2. *Paper-chromatography of the oligosaccharides present in the extracts.* The extracts from all three plants gave similar chromatograms, developed during 48 h by solvent A: Fraction 0, sucrose ($R_S = 1.00$), fraction I ($R_S = 0.61$), fraction II ($R_S = 0.31$), fraction III ($R_S = 0.27$). "Streaking" indicated the presence of saccharides moving more slowly than fraction III.

A 10 % solution of each extract (1.8 ml) was mixed with acetate buffer of pH 5 (0.2 ml). Invertase concentrate BDH (3 drops) and a few drops of toluene were added and the reaction mixture left for 24 h at room temperature. The enzymic hydrolysate was examined by chromatography using solvent B. All the oligosaccharide spots (fractions 0 to III) had disappeared whereas the quantity of fructose and glucose was substantially increased. It was not possible to detect spots of melibiose (which would correspond to the trisaccharide raffinose degraded by invertase).

3. *Separation and extraction of the oligosaccharide fractions I, II, III.* The separation was achieved by chromatography by solvent B on thick paper (Whatman 3 MM). The

paper sections were extracted twice by methanol at room temperature. After removal of the methanol at reduced pressure the following yields of crude fractions were obtained: *Galanthus Elwesii*: I, 0.54 g, II, 0.30 g, III, 0.20 g. *Leucojum vernum*: I, 0.55 g, II, 0.49 g, III, 0.27 g. *Leucojum aestivum* I, 1.20 g, II, 0.57 g, III, 0.39 g. Extraction of a paper blank (150 g) yielded a residue of 70 mg, which apparently was not of carbohydrate character. 150 g of paper would contain approximately 250 mg of the crude oligosaccharide fractions.

Purification of the fractions could be obtained by filtration of the crude sugars in aqueous solution through a filter loaded with charcoal. The present work is limited to the trisaccharide fractions (I).

4. *The trisaccharide fractions (I)*. The purified fractions I had an optical rotation $[\alpha]_D + 18^\circ$ (c 1, water). The molecular proportion glucose:fructose was found to be nearly 1:2 after complete hydrolysis with invertase, *Galanthus Elwesii* 1:2.06, *Leucojum vernum* 1:1.87, *Leucojum aestivum* 1:1.88. The sugar analyses were carried out by colorimetry (anthrone reagent) after separation of the components on chromatograms.¹¹

Paper chromatography by solvent C is known to separate 6-kestose from 1-kestose and neokestose. Fractions I from the three plants could be clearly distinguished from 6-kestose ($R_S = 0.44$). The trisaccharide fractions moved exactly as fast as 1-kestose and neokestose ($R_S = 0.56$) (undistinguishable).

Thin-layer chromatography using solvent D indicated that the trisaccharide fractions all consisted of a mixture of the two kestoses which were not separated by paper chromatography. The results are shown in Table 1.

5. *Methylation analysis*. The trisaccharides (10–40 mg of fractions I) were dissolved in dimethylformamide (1.25 ml) and were heated to 80°C if necessary. Undissolved material was removed by centrifuging. The solution was cooled to room temperature, and a mixture of barium oxide and barium hydroxide hydrate (25:1 w/w) was added (0.26 g), followed by the addition of methyl iodide (0.25 ml). The ground glass stoppered reaction tube was shaken for about 20 h at room temperature. After removal of solid material the reaction mixture was diluted with chloroform (5 ml) and washed with thiosulfate (0.1 N, 1 ml) and water (8 × 2 ml), successively. The chloroform solution was dried over sodium sulfate and evaporated to dryness under reduced pressure at 30°–40°C. Complete methylation was indicated by IR spectra (absence of OH-groups).

The permethylated trisaccharide fractions were hydrolyzed in 1 N sulfuric acid (1 ml) at 100°C under reflux for 4 h. After neutralisation with barium carbonate and removal of solid matter the solution was evaporated to dryness under reduced pressure at 30°–40°C. The residuum was dissolved in chloroform and subjected to thin-layer chromatography on silica gel (solvents E, F). Comparison with authentic specimens indicated the presence of 2,3,4-tri-*O*-methyl-glucose, 3,4,6-tri-*O*-methyl-fructose, 2,3,4,6-tetra-*O*-methyl-glucose and 1,3,4,6-tetra-*O*-methyl-fructose. The methyl sugars were well separated and characterized by reference samples in both solvent systems.

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