Gas-Liquid Chromatography and Mass Spectrometry of Quinoxalines Derived from Various Homoglucans by Alkaline o-Phenylenediamine Method

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Quinoxalines derived from various homoglucans by the alkaline OPD method were subjected to GLC and GC-MS analyses.

The main quinoxalines derived from amylose and curdlan were identified as 2-hydroxymethyl-3-(2',3'-dihydroxypropyl)quinoxaline (M-1) and 2-(2',3',4'-trihydroxybutyl)quinoxaline (G-1), respectively, using a 2% OV-210 column after trimethylsilylation. Two quinoxalines M-1 and G-1 were formed from barley glucan and lichenan, and the approximate molar ratios of the 1,4- to 1,3-linkage in both glucans were estimated to be 1.6 and 2.2, respectively. From scleroglucan, G-1 and 2-(2',3'-dihydroxy-4'-D-glucopyranosyl)quinoxaline were produced, reflecting a 1,3- and branched 1,6-linkage in the glucan. The ratios of the 1,4- to 1,3-linkage of barley glucan and lichenan, or the 1,6- to 1,3-linkage of scleroglucan estimated from the formed quinoxalines were in accordance with those obtained by conventional methylation analysis.

Quinoxaline formation from carbohydrates has already been investigated and a method for the analysis of various carbohydrates has been described.1~5) When carbohydrate is treated with excess o-phenylenediamine (OPD) and Na₂SO₃ in an alkaline buffer and heated to 100°C under deoxygenated conditions, the first step of the alkaline degradation of carbohydrate is the formation of dicarbonyl compounds specific to the structure of the mother substance, followed by the formation of various kinds of quinoxalines.

For the analysis of these quinoxalines, various chromatographic techniques such as ion-exchange chromatography,2,3) thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC)6,7) have been investigated. The TLC analysis of quinoxaline was found to give a good separation.8,9) However, for a quantitative analysis, the TLC method does not satisfy the requirements. Most of these quinoxalines contain more or less hydroxyl residues in their side chains which are derived from the original sugars.10) If we can distinguish and determine the structures of the side chains of the quinoxalines formed by GLC and gas chromatography with mass spectrometry (GC-MS), we may obtain important information about the mother carbohydrate. If the carbohydrate is a glucan, the glucosidic linkage of the original glucan may give its corresponding specificity in the chromatogram.

To evaluate this alkaline OPD method for the structural analysis of homoglucans, we investigated the GLC method for our alkaline OPD method. This present investigation was also designed to establish a rapid determination of quinoxalines derived from various homoglucans using GLC and GC-MS analyses.
MATERIALS AND METHODS

Barley glucan (β-1,3, β-1,4 glucan) was a generous gift from Amano Pharmaceutical Co., Ltd. Curdlan (β-1,3-glucan) (DPn = 455) was a product from Takeda Chemical Ind. Ltd. Lichenan (β-1,3, β-1,4 glucan) was obtained from Sigma Chemical Co., and purified by alcohol fractionation after centrifugation of the aqueous solution of the glucan at 21,000 × g for 1 hr. Amylose was a partially hydrolyzed and fractionated specimen of potato starch from Pierce Chemical Co., and the degree of polymerization was 116.6, as determined by the modified Somogyi method.8 All other reagents were of analytical grade unless otherwise stated.

Experimental procedures. Conditions for the reaction of the alkaline OPD method were essentially the same as previously reported.8) For barley glucan, 5 ~ 10 mg of the glucan was dissolved in 0.2 M carbonate buffer (pH 10) containing 0.1 M OPD and 0.3 M Na2SO3, under nitrogen bubbling. Scleroglucan was suspended in the carbonate buffer, then sonicated for 30 min at 10 kHz to facilitate the solubilization. Since each glucan used had a somewhat different solubility, 1 N aqueous solution was used to dissolve the less soluble glucans, and the final pH was adjusted to 10 by adding NaHCO3. For curdlan or lichenan, the required volume of 1 N NaOH was added to the sample in an ice-cooled water bath with stirring, and 0.4 M NaHCO3 and deionized water were added to produce a final reaction mixture containing 0.2 M NaOH and 0.2 M NaHCO3. After sufficient N2 bubbling, the solution was heated in an oil bath at 100°C under anaerobic conditions. After heating for a given time, the reaction was stopped by cooling the reaction mixture with an ice-water. Two ml of the reaction mixture was extracted with an equal volume of n-butanol 3 times, using a vortex mixer. The n-butanol extracts were combined and dehydrated with anhydrous Na2SO4. After vigorous shaking of the extracts for 2 or 3 min in a glass-stoppered tube, the mixture was centrifuged at 3000 x g for 10 min. The supernatant was evaporated to dryness under reduced pressure at a bath temperature not exceeding 50°C. To remove the remaining n-butanol, the residue was again dried in vacuo in a desiccator over silica gel for 10 or 20 min. The residue was dissolved in an aliquot (0.1 ml) of pyridine containing 1 μmol of phenyl β-glucoside as an internal standard. To the solution was added 0.05 ml of hexamethyldisilazane and 0.03 ml of trimethylchlorosilane, followed by vigorous shaking for 2 or 3 min with a vortex mixer. After the mixture was treated at 50°C for 1 min, the supernatant was subjected to GLC analysis.

GLC and GC-MS analyses. GLC was carried out with a Yanagimoto gas chromatograph model GCG 550F equipped with a flame ionization detector. The conditions of GLC were as follows: a glass coil column (0.2 x 200 cm) was packed with 2% OV-210 on Gaschrom Q (100 ~ 200 mesh). The flow rate of the carrier gas (N2) was 17.5 ml/min, and the column temperature was raised from 140°C to 220°C at a rate of 2°C/min. The temperature of the detector and the injector was 250°C.

For analysis of the partially methylated alditol acetates of homoglucans, a glass column packed with 3% OV-225 on Gaschrom Q was used with a column temperature of 180°C.

GC-MS was carried out with a Hitachi RMU-6MG mass spectrometer. The GC-column was 2% OV-210, the flow rate of the carrier gas (He) was 35 ml/min, the accelerating voltage was 3.2 kV, and the column temperature was raised from 160°C to 200°C at a rate of 2°C/min. The temperature of the injector and the separator was 250°C, with the ionizing energy and current being 20 eV and 60 μA, respectively.

Methylation analysis. Methylation analysis of some homoglucans was carried out by the modified method12,13 of Hakomori.14 To 2 mg of the glucans tested was added 2 ml of distilled dimethylsulfoxide, and the suspension, cooled by ice, was sonicated at 10 kHz for 5 to 10 min to solubilize it thoroughly. To this solution, fresh methylsulfonfyl carbamion (1 ml) was added to convert to the corresponding sugar alkoxide, under a nitrogen stream. The sugar alkoxide was then methylated by the addition of methyldiisodide (0.5 ml) at 18 ~ 20°C. The methylated product was extracted with CHCl3 (20 ml × 4). The extracts were washed several times with water, dehydrated with MgSO4, and evaporated to a syrup. The methylated homoglucans were converted to their alditol acetate derivatives, according to Yamaguchi et al.15

RESULTS

GLC of authentic quinoxaline derivatives

The conditions for separation of the quinoxaline derivatives were studied by GLC, using various kinds of column packing: SE-, OV-, PEG-series, etc. Figure 1 shows a gas chromatographic pattern of the authentic quinoxaline derivatives. Under the conditions of chromatography, these quinoxaline derivatives were well separated from each other. The isolation and identification for most of the quinoxalines have previously been reported.8 Some of them are still not described, and these will be treated elsewhere.

The calibration curve of the peak height
response was linear over the working range of 0.2 to 2 \( \mu \)mol per 0.18 ml of the reaction mixture.

**Quinoxalines derived from amylose and curdlan**

Figure 2 shows the gas-chromatographic separation of quinoxalines derived from amylose and curdlan. As is evident from this figure, each glucan showed only one predominant peak \( a \) or \( b \), except for that of the internal standard, although other minor peaks due to non-reducing terminal glucose can be seen. GC-MS analysis of the TMS-derivatives of the quinoxalines is shown in Fig. 3. Considering the GLC retention time and the fragment ions of GC-MS, peak \( a \) derived from amylose was

![Fig. 1. Gas-Liquid Chromatogram of Authentic Quinoxaline Derivatives as Their Trimethylsilyl Ethers.](image)

![Fig. 2. Gas-Liquid Chromatograms of Quinoxalines Derived from Amylose (A) and Curdlan (B).](image)

![Fig. 3. Mass Spectra of Peak a and b in Fig. 2.](image)
identified as quinoxaline M-1, whereas peak b from curdlan was identified as quinoxaline G-1.

This indicates that α-1,4 glucan produces mainly quinoxaline M-1, due to the alkaline degradation products, together with β-1,3 glucan quinoxaline G-1.

Quinoxalines derived from lichenan and barley glucan

Figure 4 shows the results for lichenan and barley glucan. Each glucan produced two major peaks (peaks a and b), showing the corresponding retention times as those of the quinoxalines M-1 and G-1. The molar ratio of these quinoxalines G-1 and M-1 derived from lichenan was estimated to be 1 : 1.56, whereas that from barley glucan was 1 : 2.22. These results agreed well with those obtained from the methylation analysis described below.

Quinoxalines derived from scleroglucan

A thin-layer chromatogram of the quinoxalines derived from scleroglucan showed two spots with Rf values of 0.76 (I) and 0.61 (II) by the solvent system of methyl ethyl ketone–acetic acid–water (3 : 1 : 1). The former spot (I) coincided with that of quinoxaline G-1, together with its colorization, while the latter (II)
Quinoxalines from Various Homoglucans

seemed to be a larger quinoxaline having a glucose residue in its side chain, because of its slow mobility.8*

Figure 5 shows the gas-chromatographic separation of the quinoxalines derived from scleroglucan. Two main peaks, A and B, were observed. Peak A was identified as quinoxaline G-1, both from the retention time, and from the characteristic fragment ions of GC-MS. Peak B was subjected to GC-MS analysis as shown in Fig. 6. Characteristic fragment ions were observed at m/z 204 (TMSO-CH=CHOTMS), 361 (M+ - TMSO-glucose), and 739 (M+-TMSO). These fragment ions agreed with those of quinoxalines obtained from gentiobiose and partially hydrolyzed dextran (Q-D-l).8) From these results, the structure of peak B was determined to be:

\[
\text{CH}_2-\text{CH} = \text{CH2O-Glc}
\]

Methylation analysis of barley glucan, lichenan and scleroglucan

To evaluate the molar ratio of quinoxaline obtained from the OPD method, barley glucan, lichenan, and scleroglucan were methylated and analyzed, and the results were compared, as shown in Table I. The molar ratio of 1,4- to 1,3-linkage of barley glucan was 2.5, and that of lichenan was 1.5, whereas the molar ratio of the 1,3- to 1,6-linkage of scleroglucan was 2.2. These results essentially agreed with those obtained by the existing alkaline OPD method.

| Table I. GC Estimation of Linkage Ratio in Some Glucans |
|-----------------|-----------------|-----------------|-----------------|
|                 | G-1 (1,3-)      | M-1 (1,4-)      | Q-D-1 (1,6-)    | Ratio of 1,4- or 1,6- to 1,3-linkage |
|                 | (μmol)*         |                 |                 | OPD Method | Methylation |
| Lichenan        | 1.48            | 2.31            |                 | 1.6        | 1.5         |
| Barley glucan   | 0.63            | 1.40            |                 | 2.2        | 2.5         |
| Scleroglucan    | 0.94            | 0.69            |                 | 0.42       | 0.45        |

* These values were calculated as μmol using a calibration curve of the peak height response by GLC analysis.
which exhibited these peaks were most probably derived from quinoxaline M-1. Quinoxalines derived from carbohydrates in alkaline media are generally stable, except for M-1 which tends to convert to 2-(2',3'-dihydroxypropyl) quinoxaline or 2-(2',3'-dihydroxypropyl)- 3-methylquinoxaline, when heated for a long period in an alkaline solution. Details of the conversion of M-1 will be treated elsewhere.

A quinoxaline having one whole glucose residue in its side chain structure should be formed, when the alkaline degradation proceeds along the main α-1,4 glucosidic chain of amyllose, if there is a glucose unit with an α-1,6 linked glucose residue as a branch, in a similar manner to scleroglucan. The determination limit of these minor components of carbohydrates, i.e., α-1,6 linked branches, by the present alkaline OPD method may depend on the pretreatment of the sample, followed by the use of separation techniques for quinoxaline derivatives by GLC analysis. Consequently, the limit will be discussed after more data have been obtained by repeated experiments. Regarding the limits of applicability, we consider that one 1,6-linked branch-glucose residue will be detectable with the present technique, if such a branch is present in a linear amyllose chain with about 30 glucose residues. This can be predicted from the results shown in Fig. 5, where the 1,6-linked glucose residues are converted to the side chains of the corresponding quinoxaline.

As barley glucan and lichenan consist of both β-1,3- and β-1,4-linkages, the formation of the quinoxalines G-1 and M-1 was to be expected. In the alkaline OPD method, the ratio of quinoxaline G-1 to M-1 is taken to be the same as the ratio of the 1,3- to the 1,4-linkages of the glucans. Our results show that the ratio of quinoxaline G-1 to M-1 derived from lichenan was about 1 : 1.5. The molar ratio of this quinoxaline is somewhat smaller than the reported molar ratio of 1 : 2, possibly due to the origin of the lichenan used. This result suggests that the repeating unit of the structure is not strictly regular in this glucan.

Quinoxaline G-1, having one additional glucose residue at C-4' in the form of ether, was obtained from scleroglucan (Fig. 5). When isomaltose and gentiobiose were treated with OPD, the same GLC and GC-MS patterns as those in the case of scleroglucan were obtained. Thus, the present method seems to be convenient for detecting the side-chain structures of branched polysaccharides.

By using the alkaline OPD method, scleroglucan was estimated to contain one mol of 1,6-linked side chains per 2.5 mol of main chain glucose residues, showing a fairly good agreement with the results of other investigators.

From the results above, we propose a simple and rapid method for the structural analysis of homoglucans with 1,3-, 1,4-, 1,3- & 1,4-, and 1,3- & 1,6-linkages, and with branched sugar residues at the position of C-6, using GLC analysis. Furthermore, this alkaline OPD method can be emphasized to be able to distinguish the branched chain from the main chain, and its ratio to the main chain sugar residues.

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REFERENCES

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Quinoxalines from Various Homoglucans