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# GEL PERMEATION CHROMATOGRAPHY OF MALTOSACCHARIDES ON POLYACRYLAMIDE GEL

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## SUMMARY

Gel permeation chromatography of maltooligosaccharides of various structures has been carried out on a column of Bio-Gel P-2 and on a multicolumn system of Bio-Gel P-2 and P-6 at elevated temperature. The compounds examined are linear and singly branched maltooligosaccharides prepared enzymatically from starch, multiply branched oligosaccharides derived from pullulan and cyclodextrins. The oligosaccharides are each eluted from the column at a specific value of relative elution volume. The methods described are applicable to the quantitative determination of oligosaccharides, and to the preparation of linear and branched oligosaccharides.

## INTRODUCTION

We have been working on the separation and the determination of maltosaccharides by improved carbon column chromatography[1] and gas–liquid chromatography[2]. These studies are related to our work on the action patterns of $\alpha$- and $\beta$-amylase on various substrates of different structures[3,4], and to work on the specific oligosaccharide-producing amylase[5]. John *et al.*[6] developed an excellent technique of fractionating maltosaccharides on a column of Bio-Gel P-2 at elevated temperature. Several studies on the fractionation of oligosaccharides using this system have since been reported[7–10].

In this paper, we summarize our results on the fractionation of maltooligosaccharides of various structures (linear, singly branched, multiply branched and cyclic) by gel permeation chromatography (GPC) on Bio-Gel P-2 and P-6.

## EXPERIMENTAL

### *Acrylamide gels*

Bio-Gel P-2 (minus 400 mesh, control number 118554) and Bio-Gel P-6 (minus 400 mesh, control number 11656) were purchased from Bio-Rad Labs., Richmond,

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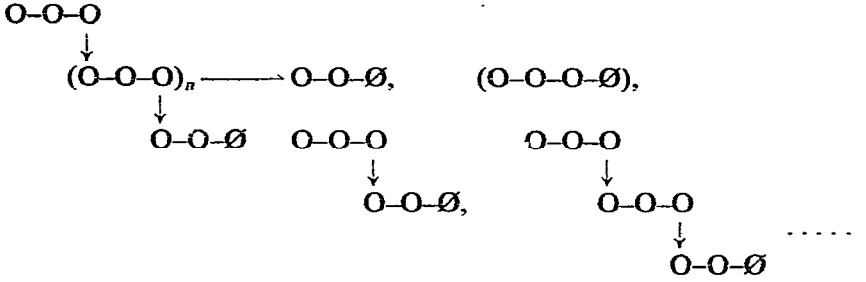
Calif., U.S.A. The gels were passed through a standard sieve (250 mesh) in order to obtain a uniform particle size.

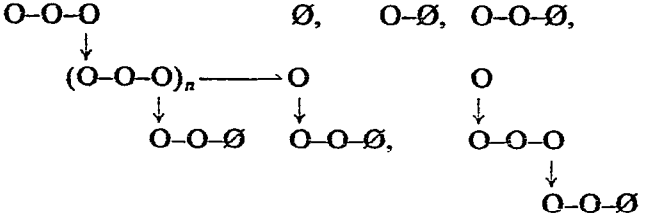
*Maltooligosaccharides*

Linear maltooligosaccharides were obtained by the coupling reaction of *Bacillus macerans* cyclodextrin glycosyltransferase with α-cyclodextrin and maltose[11]. Most of the unreacted α-cyclodextrin was removed by shaking with tetrachloroethane. Maltohexaose-producing amylase digests were obtained by the action of *Aerobacter aerogenes* amylase[5] on starch and β-limit dextrin of amylopectin. A series of non-reducing end glucosyl stubbed oligosaccharides (starting with panose) were obtained[3] from lintnerized potato starch[12].

Multiply branched oligosaccharides, prepared by the action of pullulanase, β-amylase and pullulanase on pullulan, were kindly supplied by Dr. Enevoldsen, Copenhagen. The following structures result[13] from the action of pullulanase on pullulan*:



The action of pullulanase and β-amylase on pullulan yields the structures:



α-, β- and γ-cyclodextrins were prepared by the action of *B. macerans* cyclodextrin glycosyltransferase on soluble starch[14]. The whole digest was then treated with glucoamylase in order to hydrolyze linear and branched oligosaccharides to glucose[15].

*Enzymes*

*B. macerans* cyclodextrin glycosyltransferase was prepared according to the

* Symbols and abbreviations: $G_1$, $G_2$, $G_3$, etc. = glucose, maltose, maltotriose; $B_4$ and $B_5$ = singly branched oligosaccharides of DP (degree of polymerization) 4 and 5, $BB_7$, $BBB_{10}$, $BBBB_{13}$, etc. = multiply branched oligosaccharides; O = D-glucose unit; —, α-1,4 bond; ↓, α-1,6 bond; Ø = reducing terminal D-glucose unit.

method of Tilden and Hudson[16]. Maltohexaose-producing amylase was prepared as previously described[5]. *Rhizopus niveus* glucoamylase was purchased from Seikagaku Kogyo, Tokyo, Japan (Cat. No. G 0561) and soybean $\beta$-amylase was a gift from Dr. T. Komaki, Nagase Co. Crystalline *A. aerogenes* pullulanase prepared according to the method of Wallenfels *et al.*[17], was purchased from Hayashibara Biochem. Res. Lab., Okayama, Japan.

*Preparation of the column*

Bio-Gel P-2 and P-6 gels were allowed to swell in boiling water for 2 h and were then packed into a glass column (100 × 2.65 mm) with a warm water jacket (55°). For this purpose, Excel upflow and downflow columns (Shoei Seisakusho, Tokyo, Japan) were used. The swollen gel was slowly packed into a column using a gel applicator, in order to avoid irregular packing at the bottom of the column.Two columns of Bio-Gel P-2 were connected in series (length, 2 m) in order to fractionate oligosaccharides of DP lower than 12. Oligosaccharides having DP values greater than 12 were fractionated by consecutive gel permeation on Bio-Gel P-6 and P-2 as shown in Fig. 1. We did not observe any significant difference between the resolution of oligosaccharides on columns connected in the downflow–upflow and upflow–upflow modes. The column was eluted by degassed de-ionized water by use of a peristaltic pump (flow-rate, 15–32 ml/h).

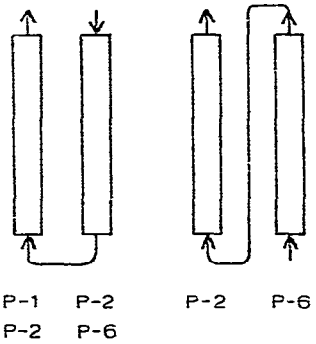



Fig. 1. Connections for the multicolumn system. Each column (100 × 2.65 cm) was jacketed with water at 55°.

*Analytical procedures*

All the analytical procedures were carried out by a Technicon AutoAnalyzer. The eluate from the column was detected by one of the following methods. The first method is automatic detection connected directly to the AutoAnalyzer. One sixth of the eluate was introduced into the analytical line and the remainder was collected by a fraction collector. A schematic flow diagram of the AutoAnalyzer is shown in Fig. 2. The oligosaccharides were determined by the orcinol–sulphuric acid method[18]. As shown in Fig. 2, after the Bio-Gel column, one line is connected to the proportioning pump of the AutoAnalyzer and the other is connected to a fraction collector (F.C.). In the second method, all of the eluate was introduced directly into a fraction collector, and then assayed through the "Sampler" of the AutoAnalyzer. This method is mainly used for slow flow-rates in order to save reagents. Instead of running the AutoAnalyzer for 40 h, all of the samples up to $G_1$ were assayed within several hours.

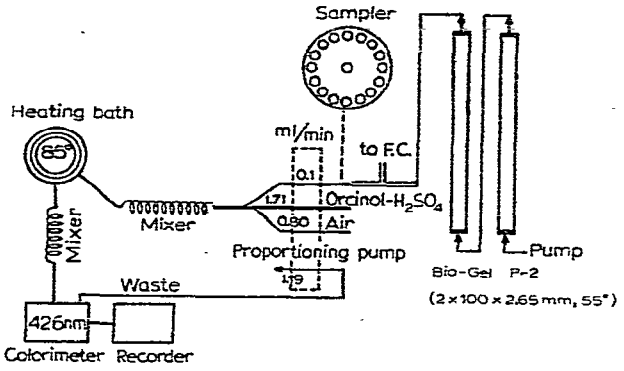



Fig. 2. Flow diagram of the columns and the Technicon AutoAnalyzer. For details of column elution see the text. F. C. = Fraction collector.

Paper chromatography was carried out[3] in order to verify the purity of the fractions.

## RESULTS AND DISCUSSION

As mentioned by John *et al.*[6], a column of Bio-Gel P-2 fitted with a jacket at elevated temperature gives a satisfactory resolution of linear maltooligosaccharides. Here we discuss our results for maltooligosaccharides of different structures, obtained on columns of Bio-Gel P-2 and P-6.

### *Resolution of linear oligosaccharides*

In order to determine the basic conditions for the column, various amounts of oligosaccharides were injected on to the column. The column was then eluted at different flow-rates between 15 and 35 ml/h. As shown in Fig. 3, there was no difference in the elution volume of oligosaccharides when the amounts injected were between 50 and 200 mg. The poor resolution between $G_5$ and $G_6$ was caused by interference from unreacted $\alpha$-cyclodextrin which was eluted just before $G_5$. The peak

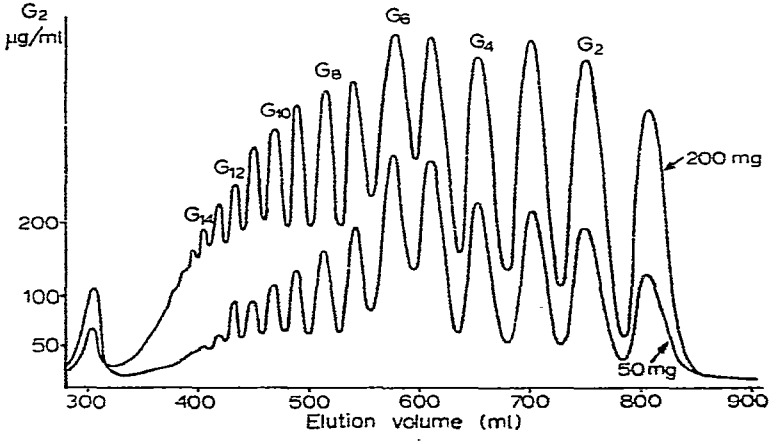



Fig. 3. Elution profile of linear maltooligosaccharides on a 2-m column of Bio-Gel P-2.

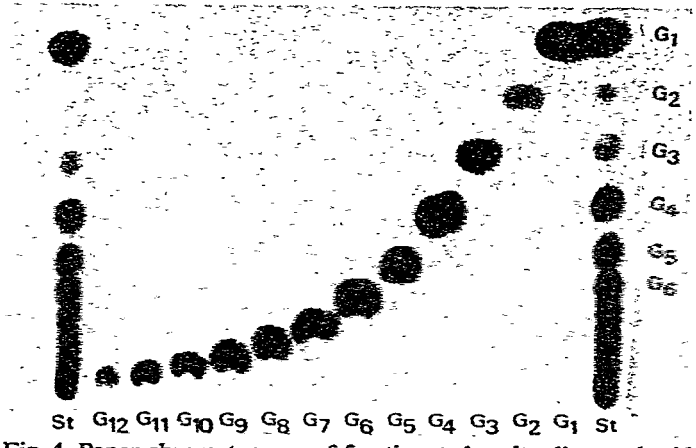



Fig. 4. Paper chromatogram of fractionated maltooligosaccharides.

areas for different DP values were collected, and the purity was controlled by paper chromatography (Fig. 4).

We obtained oligosaccharides up to DP 12–13 in high purity using the Bio-Gel P-2 column. For further experiments, amounts of sample of up to 400 mg were injected on to the column which was eluted with degassed de-ionized water at a flow-rate of 32 ml/h.

*Maltohexaose-producing amylase digest of starch and β-limit dextrin*

This separation was employed in order to separate $G_6$ from the maltohexaose-producing amylase digest. Fig. 5 shows an elution profile of the maltohexaose-

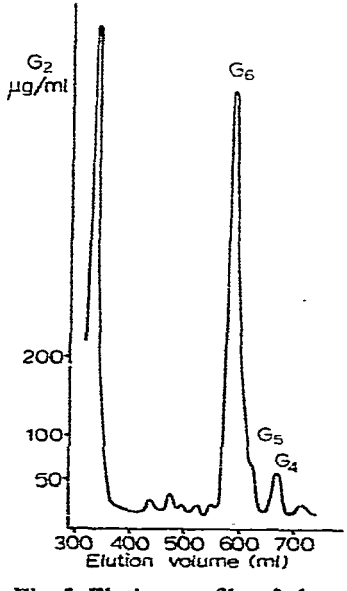



Fig. 5. Elution profile of the maltohexaose-producing amylase digest of starch on a Bio-Gel P-2 column.

producing amylase digest of starch in which the very large peak of $G_6$ is overlapping the small peak of $G_5$ which appears as a shoulder. The elution of branched oligosaccharides, which were prepared by the anomalous reaction of maltohexaose-producing amylase on $\beta$-limit dextrin, is shown in Fig. 6. The structures of the fractionated $B_6$, $B_7$ and $B_8$ have been determined and are discussed elsewhere[19]. They are oligosaccharides which possess one $\alpha$-1,6-glucosidic linkage.

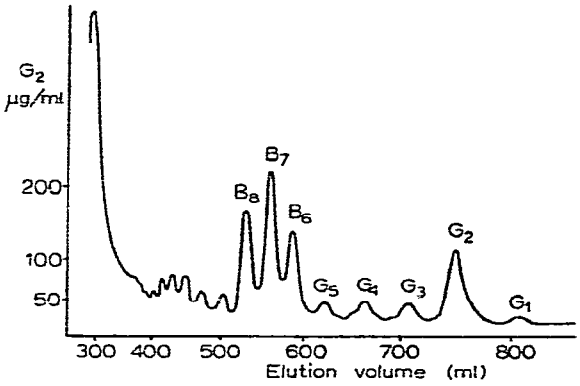



Fig. 6. Elution profile of the maltohexaose-producing amylase digest of amylopectin $\beta$-amylase limit dextrin on Bio-Gel P-2.

*Multiply branched oligosaccharides derived from pullulan*

The multiply branched oligosaccharides derived from pullulan was fractionated on the Bio-Gel P-2 column. The series $G_3$, $G_4$, $B_6$, $BB_9$, $BBB_{12}$, $BBBB_{15}$ and $BBBBB_{18}$ were clearly separated by a P-2 column up to DP 18 (Fig. 7). Another series, $G_1$, $G_2$, $G_3$, $B_4$, $BB_7$, $BBB_{10}$, $BBBB_{13}$ and $BBBBB_{16}$, was separated on the same column, and higher oligosaccharides were eluted as a large broad peak (Fig. 8). The same sample was passed through the combination of columns of P-6 and P-2 (Fig. 9), and oligosaccharides up to DP 25 were clearly separated. Figs. 8 and 9 indicate the usefulness of the combination of columns for mixtures having a wide range of DP.

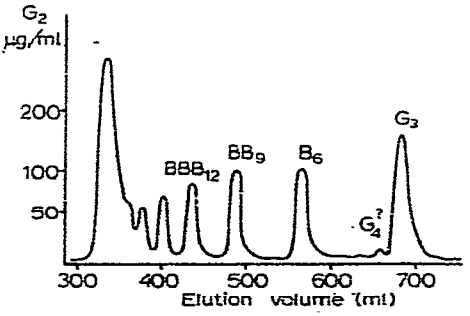



Fig. 7. Elution profile of a pullulanase hydrolyzate of pullulan on a Bio-Gel P-2 column.

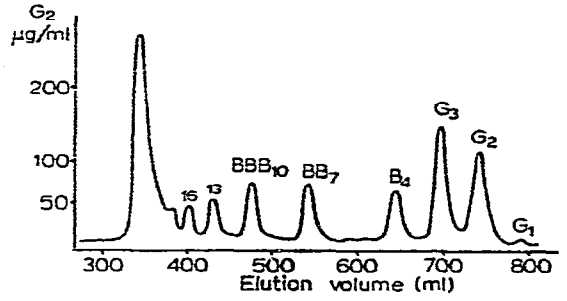



Fig. 8. Elution profile of a pullulanase and β-amylase hydrolyzate of pullulan on Bio-Gel P-2.

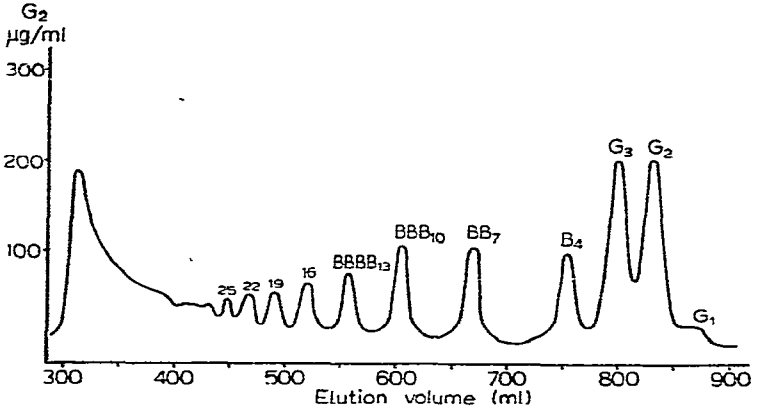



Fig. 9. Elution profile of a pullulanase and β-amylase hydrolyzate of pullulan fractionated by the multicolumn system of Bio-Gel P-6 and P-2. The size of each column was 100 × 2.65 cm.

*α-, β- and γ-cyclodextrins*

Cyclodextrin[14] is a general name for maltodextrin having a cyclic conformation. α-, β- and γ-cyclodextrins are cyclohexa-, cycloheptа- and cyclo-octaamylose, respectively. These dextrins have specific characteristics when compared with linear oligosaccharides of the same molecular weight. Glucoamylase-treated cyclodextrin glycosyltransferase digest was injected on to the P-2 column and eluted in the usual manner. The three different cyclodextrins were separated together with a large peak due to glucose formed by the action of glucoamylase on linear and branched oligosaccharides (Fig. 10).

*Relation between the specific elution volume and the molecular weight of maltooligosaccharides*

The separation of substances of high molecular weights on the Bio-Gel column is considered to be based on the exclusion principle, where only small molecules can diffuse into the network of the gel matrix. As shown in the figures, maltooligosaccharides of different structures were eluted at the specific elution volume. In order to determine the relation between the molecular shape and the permeability of the gel matrix to the oligosaccharides, molecular weights and relative elution volumes

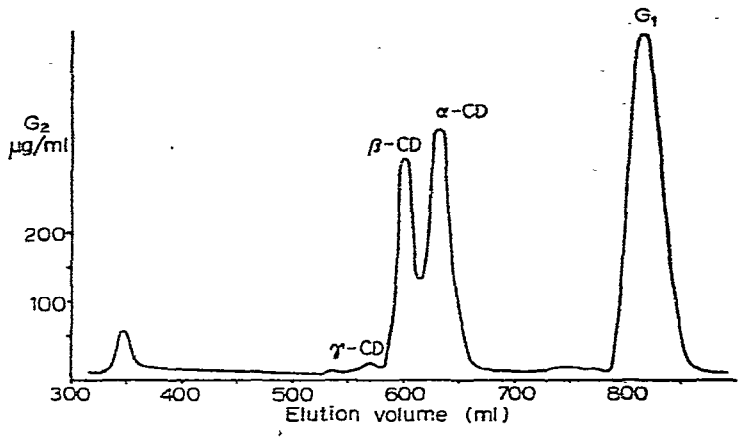



Fig. 10. Elution profile of cyclodextrins (CD) and glucose on a Bio-Gel P-2 column.

were calculated (Fig. 11) from the data for the linear maltosaccharides (Fig. 3), for the non-reducing end glucosyl stubbed maltosaccharides (O(↓)–O–O–···Ø), for the multiply branched oligosaccharides derived from pullulan (Figs. 7 and 8) and for the cyclodextrins (Fig. 10).

As seen in Fig. 11, each series of oligosaccharides has a specific relation between the molecular weight and the relative elution volume, $V_e/V_0$. Oligosaccharides

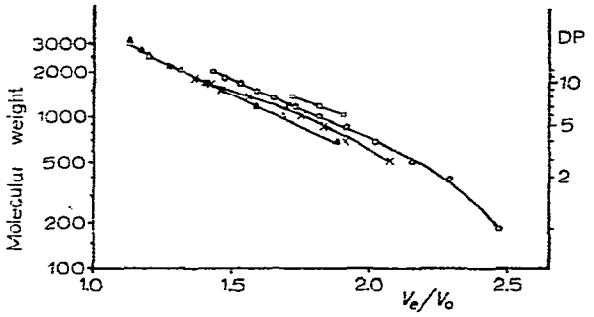



Fig. 11. Relation between $V_e/V_0$ and the molecular weight of maltooligosaccharides of different structures: ○, O–O–O—Ø; △, O–O–O(↓)–(O–O–O)$_n$–O–O–Ø; where $n \geqslant 0$; □, cyclodextrin; ×, O(↓)–O–O—Ø; ▲, O(↓)–(O–O–O)$_n$–O–O–Ø; where $n \geqslant 0$.

having the same DP but of different structure had completely different elution volumes.

For example, $\alpha$-cyclodextrin and $B_4$ (O–O–Ø) had almost the same volume, followed by $G_5$. The relation between the molecular weight and $V_e/V_0$ was linear for linear oligosaccharides having DP 4–12, and for non-reducing end glucosyl stubbed oligosaccharides having DP 6–10. The relation was also linear for 6–10 $\alpha$-, $\beta$- and $\gamma$-cyclodextrin. The two series of multiply branched oligosaccharides derived from pullulan could be plotted on the same straight line.

Generally, the branched oligosaccharides were eluted earlier than the linear molecules on the Bio-Gel P-2 column. From the results of Fig. 11, we conclude that the order of elution from the Bio-Gel P-2 column is multiply branched, singly branched, linear and cyclic oligosaccharides of the same molecular weight.

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