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QUANTITATIVE ETHER CLEAVAGE OF LIGANDS IN HYDROPHOBIC AGAROSES —PRECISE DETERMINATION OF THE DEGREE OF SUBSTITUTION

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SUMMARY

The ether-linked hydrophobic substituents of octylagarose and phenylagarose were cleaved with boron tribromide and quantified by gas chromatography. The ability of boron tribromide to break ether bonds quantitatively was demonstrated by cleavage of model ethers. The procedure is sensitive, reproducible and can easily be applied to other ether-linked ligands.

INTRODUCTION

Hydrophobic agarose derivatives are frequently used for the immobilization of proteins[1] and protein separations[2] in hydrophobic chromatography. However, published results are often difficult to reproduce because the degree of substitution of the gels used is either given inaccurately or is even unknown. The ligand density of the hydrophobic substituents, however, is an important parameter in the chromatographic separation process. Hence there have been several attempts[3–5] to determine the degree of substitution on the polysaccharide matrix exactly, but all of these methods have disadvantages and are often not really reliable, especially at low ligand concentrations[6].

This paper describes a precise procedure for determining ligand densities in *n*-octyl- and phenylagarose. The basis of the method is the scission of the ether bond linking substituents to the polymeric matrix and the subsequent gas chromatographic analysis of the resulting fragments, a technique which is in principle not new and has been used in cellulose ether analysis with good results[7].

In contrast to the usual procedure with hydroiodic acid, boron tribromide was used to achieve rapid cleavage under mild conditions[8]. This type of reaction yields, in addition to less interesting brominated derivatives of the original spacer, derivatives of the hydrophobic substituent that can easily be detected and quantified by gas chromatography.

This determination is valid in a quantitative sense if one can be sure of complete ether cleavage from the polymeric matrix. For this reason two model compounds, 1-(2-hydroxyethoxy)-3-octyloxypropanol-2 and 1-(2-hydroxyethoxy)-3-

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phenoxypropanol-2, simulating the interesting ether linkages were cleaved with boron tribromide, followed by quantitative analysis of the fragments obtained.

EXPERIMENTAL

Materials

1-Bromooctane, 2-bromooctane, *n*-dodecane, octanol-1, phenol and *n*-undecane were purchased from Merck (Darmstadt, G.F.R.) and agarose (Sepharose CL 4B), octylagarose (octyl-Sepharose CL 4B) and phenylagarose (phenyl-Sepharose Cl 4B) from Pharmacia (Uppsala, Sweden). Boron tribromide and phenyl glycidyl ether were purchased from Fluka (Buchs, Switzerland), 4-bromooctane from EGA Chemie (Steinheim, G.F.R.) and silver carbonate from Degussa (Frankfurt, G.F.R.). Solvents were obtained from Merck or Riedel de Haën (Hannover, G.F.R.) and were of analytical-reagent grade.

Analysis

Detection and quantitative analysis were carried out with a Shimadzu GC 3 BT gas chromatograph and a connected Sigma 1 data system (Perkin-Elmer, Norwalk, CT, U.S.A.) on a 4.70 m × 4 mm I.D. glass column packed with 0.5% OV-17 and 7% Apiezon L on Chromosorb W AW DMCS (80–100 mesh). The oven temperature was 180°C for the octyl and 160°C for the phenyl system, and the helium flow-rate was 48 ml/min. A flame-ionization detector was used. Identification was achieved by use of reference substances or by direct coupling of the column outlet to a mass spectrometer.

Model compounds

1-(2-Hydroxyethoxy)-3-octyloxypropanol-2 (model ether I) and 1-(2-hydroxyethoxy)-3-phenoxypropanol-2 (model ether II) were synthesized from phenyl glycidyl ether and octyl glycidyl ether, respectively, and ethylene glycol according to a modified procedure of Ellingboe *et al.*[9]. Octyl glycidyl ether was prepared as described elsewhere[10].

Cleavage of model compounds

To 1 mmol of model ether I in a 5-ml measuring flask were added 600 µl of dichloromethane. The flask was held in a water-bath at 30°C while while 2 mmol of boron tribromide in 1.5 ml dichloromethane were added. After 5 min the sample was hydrolysed with 10% sodium hydroxide solution (5 × 100 µl). The strongly acidic reaction mixture was neutralized with 3.5 mmol of silver carbonate to achieve a single-phase system. As the internal standard, 0.5 mmol of *n*-dodecane in 300 µl of acetone were added and the flask was filled with acetone to the 5-ml mark.

Model ether II was treated in the same manner but the reaction time with boron tribromide had to be prolonged to 60 min; 0.54 mmol of *n*-undecane was used as the internal standard.

Standards for quantification of model ether cleavage

For quantification of model ether I fragments, an acetone solution (5 ml) containing 0.5 mmol of octanol-1, 0.5 mmol of 1-bromooctane, 0.25 mmol of 2-

bromooctane, 0.025 mmol of 4-bromooctane and 0.5 mmol of *n*-dodecane was used. The standard for model ether II (5 ml) contained 1 mmol of phenol and 0.54 mmol of *n*-undecane in acetone.

Determination of ligand densities in substituted agaroses

Octylagarose was washed several times with water to remove stabilizers and was allowed to settle in a measuring cylinder overnight. The gel volume was subsequently corrected to exactly 1 ml, transferred to a small-volume glass filter funnel and washed into the dichloromethane phase as described elsewhere[11]. Complete removal of acetone should be ensured (GC), because even small amounts can cause, after reaction with boron tribromide, undesirable signals in the chromatogram.

For cleavage, the material was placed in a 5-ml measuring flask together with 600 µl of dichloromethane and kept in a water-bath at 30°C, then 1.17 mmol of boron tribromide in 500 µl of dichloromethane were added. After reaction for 5 min, hydrolysis was started with 10% sodium hydroxide solution (6 × 50 µl) and, after a further 5 min, 2.5 mmol of silver carbonate was used to neutralize the acidic sample. To achieve effective mixing the flask was placed in an ultrasonic bath for a few minutes. For quantification 13.2 µmol of *n*-dodecane in 500 µl of acetone were added and the flask was filled with acetone to the 5-ml mark.

Phenylagarose was treated similarly but the reaction time with boron tribromide was prolonged to 1 h and the internal standard was *n*-undecane.

Standards for quantification of agarose derivatives

For quantification of octylagarose a solution (5 ml) containing 5 µmol of octanol-1, 20 µmol of 1-bromooctane, 10 µmol of 2-bromooctane, 1 µmol of 4-bromooctane and 13.2 µmol of *n*-dodecane in acetone was prepared. The standard for phenylagarose contained 20 µmol of phenol and the internal standard was *n*-undecane in 5 ml of acetone.

RESULTS

Model compounds

Cleavage of model ether I yielded several fragments (Fig. 1). The hydrophobic octylligand was found as isomeric 1-bromooctane (47.7%), 2-bromooctane (5.7%) and 4-bromooctane (2.8%), which were mainly formed in the first reaction step, and as octanol-1 (43.8%), a product of the hydrolysis of the boric acid ester of the second part of the reaction.

The spacer yielded 1,3-dibromopropanol-2 and 2,3-dibromopropanol-1, which formed epibromohydrin after some time. 1,2,3-Tribromopropane was also detected in small amounts. The amounts of all the octyl-containing components added up to the original molarity of the model ether and demonstrated quantitative cleavage. No unreacted model compound could be detected by gas chromatography.

Model ether II was less complex in fragmentation (Fig. 2). The chromatogram showed only phenol in addition to the already known spacer fragments.

A reaction time of 5 min did not achieve total cleavage; prolongation to 1 hour was necessary for quantitative scission.

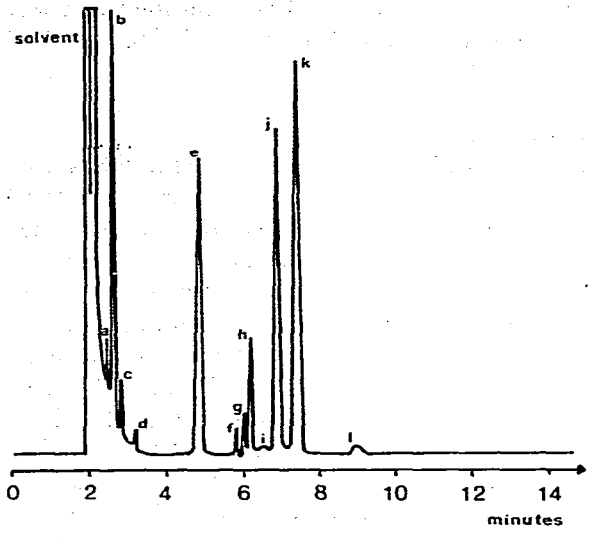

Fig. 1. Gas chromatogram of cleavage products from model ether I using BBr_3 (1:2) in dichloromethane, after hydrolysis (10% NaOH) and neutralization with silver carbonate. Peaks: a = ethylene glycol; b = 2-bromoethanol; c = epibromohydrin; d = 1,2-dibromoethane; e = octanol-1; f = 4-bromooctane; g = 2-bromooctane; h = 1,3-dibromopropanol-2; i = 2,3-dibromopropanol-1; j = 1-bromooctane; k = *n*-dodecane; l = 1,2,3-tribromopropane.

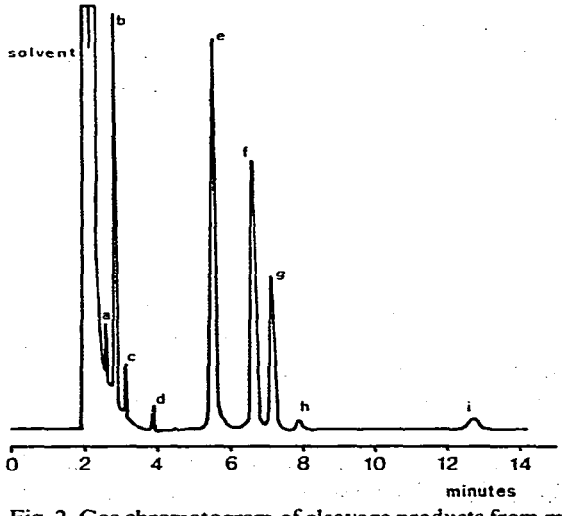

Fig. 2. Gas chromatogram of cleavage products from model ether II using BBr_3 (1:2) in dichloromethane, after hydrolysis (10% NaOH) and neutralization with silver carbonate. Peaks: a = ethylene glycol; b = 2-bromoethanol; c = epibromohydrin; d = 1,2-dibromoethane; e = phenol; f = *n*-undecane; g = 1,3-dibromopropanol-2; h = 2,3-dibromopropanol-1; i = 1,2,3-tribromopropane.

Application to agarose derivatives

For complete cleavage of model ethers, the ratio of boron tribromide to reactive groups was 1:2. In the anhydrodisaccharide unit of unsubstituted agarose nine groups are able to interfere with boron tribromide; substitution and cross-linking

would increase this number. This has to be taken in consideration when calculating the amount of reagent needed.

A series of ten samples of octylagarose were treated with boron tribromide in different ratios (Fig. 3). It could be shown that a molar ratio of at least about 1:7 (unsubstituted disaccharide to boron tribromide) was necessary to achieve optimal yields; nevertheless, an excess ratio of 1:10 was used for routine analysis. The same results were obtained for phenyl agarose.

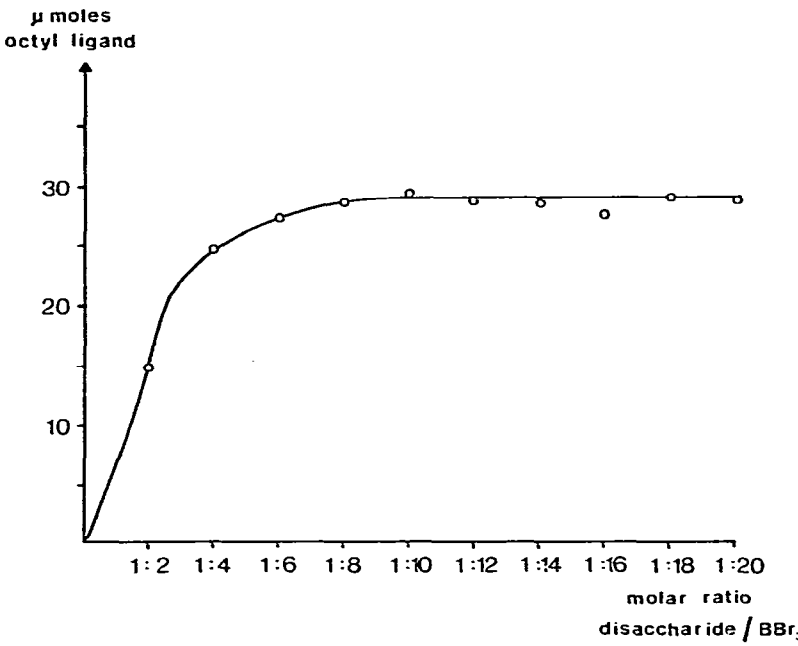

Fig. 3. Yield of octyl ligand from cleaved octylagarose as a function of anhydrodisaccharide to BBr_3 ratio. Determined by gas chromatography using an internal standard.

For both octyl- and phenylagarose the observed fragmentation patterns were the same as for the corresponding model ethers (Figs. 4 and 5), but there were some differences in their proportions.

Degree of substitution

Three different batches of commercially available octylagarose and one batch of phenylagarose were cleaved (Figs. 6 and 7) and quantified. Under the experimental conditions used no undesirable signals from polysaccharide destruction processes were observed.

With octylagarose the yield of octanol-1 was very low in contrast to the corresponding model compound. If 1.63 mmole of methanol were added to the octylagarose prior to cleavage the yield of octanol-1 was much higher and comparable to the results of model ether I cleavage; the sum of all octyl fragments remained the same, however.

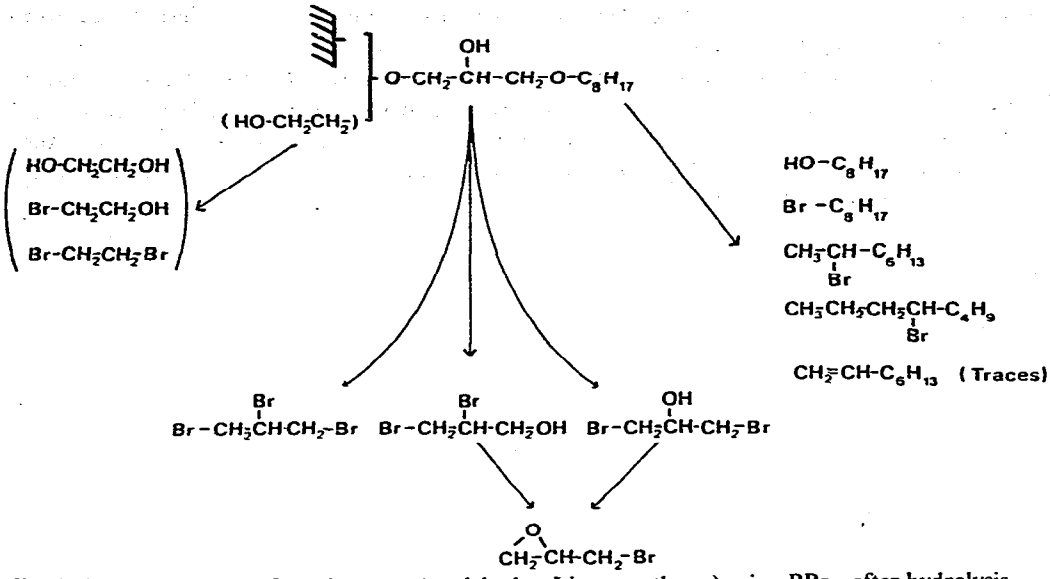

Fig. 4. Cleavage pattern of octylagarose (model ether I in parentheses) using BBr_3, after hydrolysis.

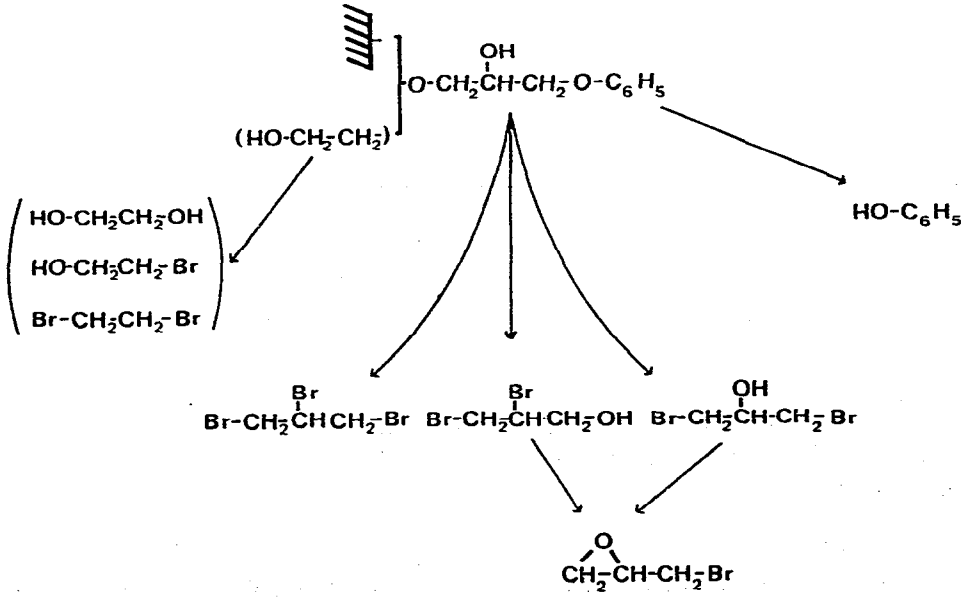

Fig. 5. Cleavage pattern of phenylagarose (model ether II in parentheses) using BBr_3, after hydrolysis.

The determined ligand densities are shown in Table I. The data were obtained as mean values from ten determinations per batch with good relative R.M.S. deviations (1.6% and 4%, for the octyl- and phenylagaroses, respectively).

Two of the octylagaroses were very similar, but one had a higher degree of substitution. In general the determined ligand densities were lower than the manufacturer's stated value of *ca.* 40 μmole/ml.

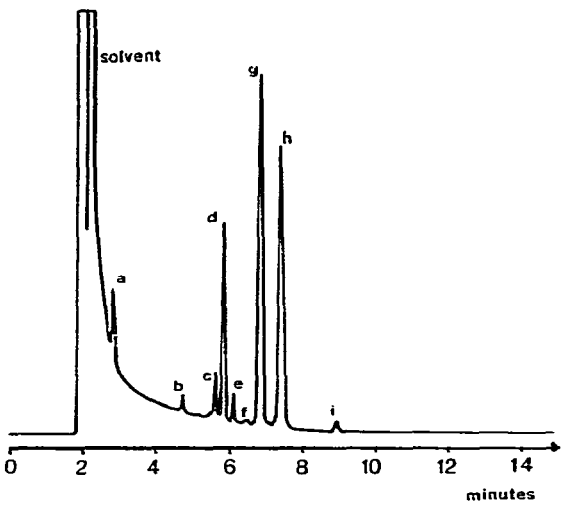

Fig. 6. Gas chromatogram of cleavage products from octylagarose (Batch 9080) using BBr_3 (1:10) in dichloromethane, after hydrolysis (10% NaOH) and neutralization with silver carbonate. Peaks: a = epibromohydrin; b = octanol-1; c = 4-bromooctane; d = 2-bromooctane; e = 1,3-dibromopropanol-2; f = 2,3-dibromopropanol-1; g = 1-bromooctane; h = *n*-dodecane; i = 1,2,3-tribromopropane.

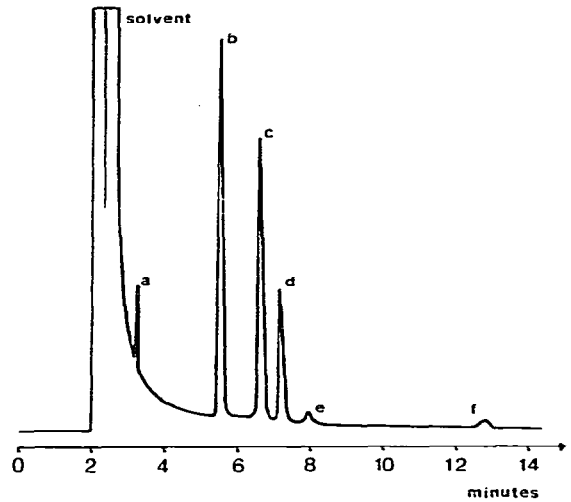

Fig. 7. Gas chromatogram of cleavage products from phenylagarose (Batch 9705) using BBr_3 (1:10) in dichloromethane, after hydrolysis (10% NaOH) and neutralization with silver carbonate. Peaks: a = epibromohydrin; b = phenol; c = *n*-undecane; d = 1,3-dibromopropanol-2; e = 2,3-dibromopropanol-1; f = 1,2,3-tribromopropane.

TABLE I

DEGREE OF SUBSTITUTION OF DIFFERENT BATCHES OF OCTYL- AND PHENYL-AGAROSE

Determination from cleavage with boron tribromide (5 min; 30°C) and subsequent hydrolysis (10% sodium hydroxide solution).

Batch No.	*Type of agarose*	*Degree of substitution (μmole/ml gel)*
9080	Octyl	29.50 ± 0.5
3375	Octyl	32.83 ± 0.5
11143	Octyl	29.62 ± 0.5
9705	Phenyl	29.19 ± 1.25

CONCLUSIONS

The described method is very sensitive and reliable even for determining low ligand concentrations of hydrophobic gels, and allows comparisons between commercial and synthesized products. It can be applied to other ligands and matrices with the same type of chemical linkage.

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