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Note

Quantitation of the ligand in Phenyl-Superose

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Phenyl-Superose™ is a new gel for hydrophobic interaction chromatography (HIC). This gel is a derivative of the cross-linked agarose gel Superose 12. The phenyl groups are coupled to the gel matrix via the reaction of phenyl glycidyl ether with Superose 12.

For the assessment of the quality of Phenyl-Superose, an accurate method for the quantitation of the ligand content is required. Three methods for the analysis of phenyl ligands in Phenyl-Sepharose CL-4B have recently been reported[1]. Two of these methods cannot be adapted to Phenyl-Superose as hydrochloric acid cannot solubilize this gel. The new cross-linking structure in Superose 12 makes this gel more resistant to acid hydrolysis[2].

This paper describes a procedure to solubilize Phenyl-Superose using boron tribromide which has successfully been used for the determination of ligands coupled to agarose gels (3–5). In this investigation the ligand cleavage products were determined by two different instrumental methods, namely UV spectrophotometry and reversed-phase chromatography. For elucidation of systematic errors the carbon content method in ref. 1 was also applied.

EXPERIMENTAL

Chemicals and apparatus

Dichloromethane, methanol, ethanol, phenol and sodium hydroxide were of p.a. quality. Boron tribromide and bromobenzene were of purum quality.

A shimadzu spectrophotometer UV-240 with a graphic printer PR-1 and a 1-cm cell was used for the spectrophotometric measurements. The high-performance liquid chromatographic (HPLC) experiments were performed on a Shimadzu LC-6A liquid chromatographic system. A Mino RPC column, 250 mm × 4.6 mm I.D. (Pharmacia, Uppsala, Sweden), was operated at 40°C. The injection volume was 25 μl and the flow-rate was adjusted to 2 ml/min. The UV detector (SPD-6AV) was operated at 270 nm and the cell volume was 8 μl.

Sample pretreatment

About 1 ml of homogenized Phenyl-Superose was transferred to a column (Pharmacia HR 5/20 glass column with one adapter). The gel was washed with 5 ml distilled water and then with 5 ml ethanol. A peristaltic pump (Pharmacia P-1) was

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used during the washing procedure and the following drying of the gel. Finally, the gel was dried at 70°C for 15 h.

Determination of the ligand content by UV spectrophotometry and liquid chromatography

About 20 mg of the dry gel were placed in a 10-ml measuring flask together with 1.3 ml dichloromethane at 25°C. The reaction was started with 700 μl of a solution of boron tribromide (1.45 mmol unless otherwise stated) in dichloromethane, which was freshly prepared every week, protected from moisture and stored at −30°C. After a reaction time of 20 min (unless otherwise stated), unchanged boron tribromide was destroyed by hydrolysis with 500 μl of 10% sodium hydroxide solution in water. The hydrolysed gel was diluted in methanol to 10.0 ml. This solution was diluted 20 times in methanol before analysis by spectrophotometry or liquid chromatography.

Spectrophotometry

The absorbance was evaluated at λ_{max} = 272 nm according to Fig. 1 (distance *h*, *i.e.* the absorbance of phenol, corrected for the blank signal; broken line in Fig. 1). The ligand density was calculated by utilizing the molar extinction coefficient, ε, of phenol, which was determinded by the addition of phenol to a solution of hydrolysed unsubstituted Superose gel (hydrolysed as Phenyl-Superose, see above). Five standard solutions of phenol in the concentration range 10–75 μM were used for the estimation of ε.

Liquid chromatography

The cleavage products phenol and bromobenzene were eluted isocratically with methanol–water (20:80 and 70:30, respectively). The standard addition method was used for the quantification of phenol and bromobenzene in the sample.

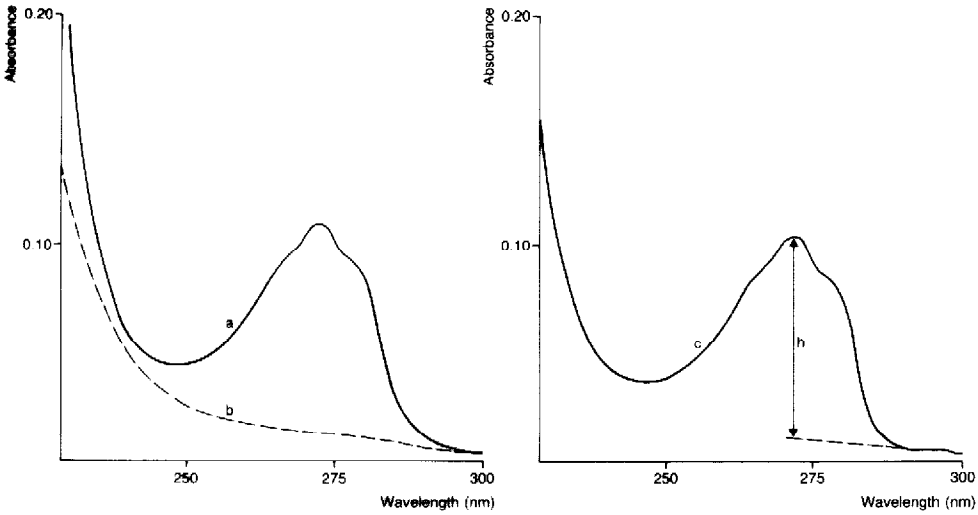

Fig. 1. UV spectra of hydrolysed Phenyl-Superose (a) and Superose (b) and of 0.05 mM phenol (c). For details see Experimental section.

Determination of the ligand content by carbon analysis

Phenyl-Superose and the corresponding unsubstituted gel were pretreated as above. The carbon content was determined in both gels, assuming that the gel is combusted to carbon dioxide. The ligand content was calculated as described.[1]

RESULTS AND DISCUSSION

The UV spectra of boron tribromide-cleaved Phenyl-Superose and of phenol (Fig. 1) suggest that the main cleavage product is phenol. However, minor amounts of bromobenzene are also expected. The molar extinction coefficient of phenol at 272 nm was estimated to $1.92 \cdot 10^3$ l mol^{-1} cm^{-1}. At the same wavelength, ε of bromobenzene is about 10 times lower.

The UV method proposed for the determination of the ligand content in Phenyl-Superose requires a quantitative detachment of the ligand and an accurate estimation of the cleavage products. The gel was treated with different concentrations of boron tribromide for various times in order to optimize the cleavage conditons (Table I). To obtain a maximum yield of phenol, 0.6 mmol of boron tribromide were required at a cleavage time of 20 min. However, the yield of phenol decreased when 1.45 mmol boron tribromide were allowed to react with Phenyl-Superose during 60 min. This decrease can be explained if the bromobenzene content increases with cleavage time. From these results (Table I) a cleavage time of 20 min and 1.45 mmol boron tribromide were chosen.

In order more closely to observe the cleavage products under the optimized cleavage conditions and to identify systematic errors, an LC method was developed. In Table II the results from the two different instrumental methods are compared. As confirmed by the LC results, the ligands were identified as phenol (99%) and

TABLE I

YIELD OF PHENOL FROM THE CLEAVAGE OF A DEVELOPMENT BATCH OF PHENYL-SUPEROSE BY DIFFERENT AMOUNTS OF BORON TRIBROMIDE AND DIFFERENT CLEAVAGE TIMES

See Experimental for a complete description of the cleavage procedure.

Amount of boron tribromide (mmol)	*Cleavage time (min)*	*Amount of phenol* (μmol/mg dry gel)*
0.415	20	0.408, 0.400
0.623	20	0.443, 0.429
1.038	20	0.426, 0.438
1.453	5	0.437, 0.435
1.453	10	0.426, 0.418
1.453	15	0.418, 0.430
1.453	20	0.434, 0.432
1.453	30	0.426, 0.430
1.453	60	0.394, 0.406
2.076	20	0.431, 0.444
3.113	20	0.430, 0.434

* Measured in accord with Fig. 1.

TABLE II

DETERMINATION OF THE LIGAND CONTENT IN PHENYL-SUPEROSE (PRODUCTION BATCHES) WITH TWO INSTRUMENTAL METHODS AND QUANTIFICATION OF BROMOBENZENE

See Experimental for a description of the sample pretreatment.

Method	*Degree of substitution (μmol/mg dry gel)*		
	Batch A	*Batch B*	*Batch C*
UV-spectrophotometry	0.62, 0.61	0.58, 0.58	0.57, 0.56
Liquid chromatography	0.57, 0.59	0.57, 0.58	0.53, 0.57
Amount of bromobenzene determined by LC	0.005, 0.005	0.005, 0.004	0.005, 0.004

TABLE III

DEGREE OF SUBSTITUTION ON DIFFERENT DEVELOPMENT BATCHES OF PHENYL-SUPEROSE DETERMINED BY TWO INDEPENDENT METHODS

Degree of substitution (μmol/mg dry gel)	
Spectrophotometry	*Carbon analysis*
0.19, 0.20	0.13, 0.16
0.40, 0.41	0.37, 0.34
0.59, 0.59	0.59, 0.62
0.67, 0.65	0.68, 0.65

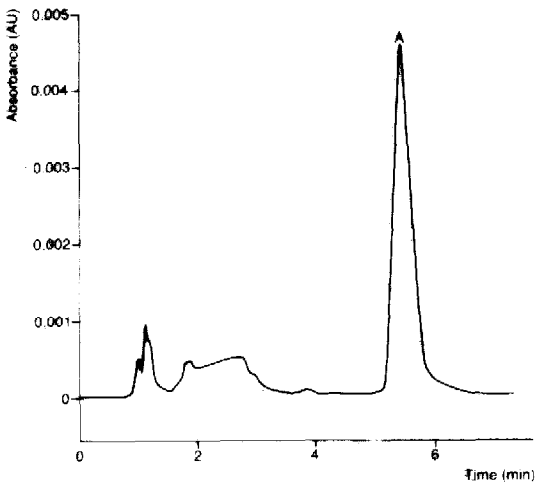

Fig. 2. Chromatogram of hydrolysed Phenyl-Superose where peak A corresponds to phenol. For details see Experimental section.

bromobenzene (1%). This demonstrates that the use of ε for phenol in the UV method results in a systematic error not greater than 1% under the optimized cleavage conditions. A representative LC chromatogram of phenol is depicted in Fig. 2.

The validity of the UV and LC methods was further elucidated by carbon analysis, a physically and chemically unrelated method. In Table III the results from the carbon analysis are compared with those from the UV method over a wide range of ligand densities. The agreement indicates that there is no evidence for systematic differences between the two sets of results.

The reproducibility of the UV method has been calculated from the analysis of 37 batches of Phenyl-Superose. The pooled standard deviation, s, was estimated as 0.01 μmol per mg dry gel were s has 39 degrees of freedom.

It can be concluded that the suggested boron tribromide cleavage produce gives a quantitative rupture of the ether bonds linking the phenyl groups to the spacer arm. In addition, the ligand is obtained as phenol (99%). Evaluation of the ligand content by determining phenol using UV spectrophotometry is therefore an accurate method. The UV method is now used in the authors' laboratory for quality control of the ligand content in Phenyl-Superose. In Table II the results are presented from three different production batches.

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