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Note

Three methods for quantitative determination of the ligand in Phenyl-Sepharose CL-4B

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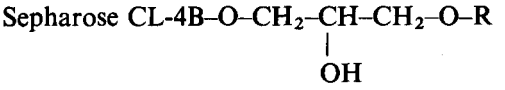
Phenyl-Sepharose CL-4B is a derivative of the cross-linked agarose gel Sepharose CL-4B. The phenyl groups are coupled to the gel matrix via the reaction of phenyl glycidyl ether with Sepharose CL-4B. The partial structure is:



where R is the phenyl group. Phenyl-Sepharose CL-4B is a gel intended for use in hydrophobic interaction chromatography (HIC), mainly for the separation and purification of proteins and peptides[1–9].

Quality control of the ligand content of different HIC gels demands simple methods with high precision and accuracy[10]. Furthermore, a knowledge of the ligand density is important for retention model studies[11]. In this paper we describe three independent methods for the quantitative determination of the ligand content in Phenyl-Sepharose CL-4B. The methods are based on UV spectrophotometry and ^{1}H NMR spectrometry and analysis of the carbon content of unsubstituted and substituted Sepharose CL-4B. Recently, Genieser *et al.*[12] described a gas chromatographic method.

EXPERIMENTAL

Chemicals and apparatus

Phenyl-Sepharose CL-4B and Sepharose CL-4B were obtained from Pharmacia, Uppsala, Sweden. Deuterium chloride and [^{2}H]dimethyl sulphoxide (DMSO-d_6; isotopic purity greater than 99.5%) were from Ciba-Geigy, Basel. Hydrochloric acid, acetone, methanol and phenol were of *pro analysi* quality, whereas 2-phenoxyethanol was of purum quality.

A Shimadzu spectrophotometer UV-240 with a graphic printer PR-1 and a 1-cm cell was used for spectrophotometric measurements. The ^{1}H NMR spectra were recorded with a Jeol Fx 200 instrument at 199.5 MHz. In the pulsed NMR experiments the number of pulses was 100, the pulse time 7 μs, the pulse delay 20 s, the acquisition time 2 s, and the delay between pulse and acquisition 50 μs. The spectral range explored was 2000 Hz.

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Sample pretreatment

About 5 ml of homogenized Sepharose CL-4B or Phenyl-Sepharose CL-4B were transferred to a glass filter funnel (G3). The gel was washed first with 5 × 5 ml of distilled water and then with 2 × 5 ml of water–acetone (1:1). The gel was shrunk with 5 × 5 ml of acetone: after each addition it was homogenized and sucked dry with a water pump. Finally, the gel was dried at 105°C for 15 h.

The degree of substitution by UV spectrophotometry

Hydrolysis and preparation of the sample solution. The dried gel (20 mg) was hydrolysed at 20°C with 1.00 ml of concentrated hydrochloric acid for 10 min. The hydrolysed gel was diluted with methanol to 10.0 ml. This solution was further diluted 20 times with methanol before the absorbance was registered at λ_{max} (270.5 nm).

Determination of the molar absorptivity of phenoxyethanol. Four standard solutions of phenoxyethanol in the concentration range 0.09–0.31 m*M* were used in the determination of the molar absorptivity (ε). In accordance with the gel sample solution phenoxyethanol was dissolved in methanol acidified with 0.06 *M* hydrochloric acid.

The degree of substitution by ^1H NMR spectrometry

The dried gel (20 mg) was hydrolysed with 200 μl of 6.3 *M* deuterium chloride at 70°C for 45 s and then cooled in an ice-bath. The sample was diluted with 2.00 ml of DMSO-d_6, and from this mixture two aliquots of 1.00 ml were taken. To one of the aliquots 100 μl of DMSO-d_6 were added, and to the other 100 μl of DMSO-d_6 containing 14.9 μmol of phenol were added. NMR spectra of these solutions were registered. The peaks from the aromatic protons were integrated, and the peak from the isotopic impurities of DMSO-d_6 served as internal standard (Fig. 1). Standard solutions of phenol, in the concentration range 4–17 m*M*, were registered in the same way. The relaxation times were measured by the inversal recovery method[13].

The degree of substitution by carbon analysis

Phenyl-Sepharose CL-4B and its corresponding unsubstituted Sepharose CL-4B were pretreated as above. The carbon content was determined in both gels.

Calculation. The amount of phenyl glycidyl ether (*X*) in milligrams coupled to 100 mg Sepharose CL-4B has been calculated from the following equation:

$$Z = \frac{100\left(Y + \frac{108.10}{150.18}X\right)}{100 + X} \tag{1}$$

Conversion of this equation gives

$$X = \frac{100(Z - Y)}{71.98 - Z} \tag{2}$$

where *Y* and *Z* are the degrees of carbon content in weight percent of Sepharose CL-4B and Phenyl-Sepharose CL-4B, respectively, 150.18 g/mol is the molecular weight of the phenyl glycidyl ether and 108.10 g/mol is the weight of the carbon

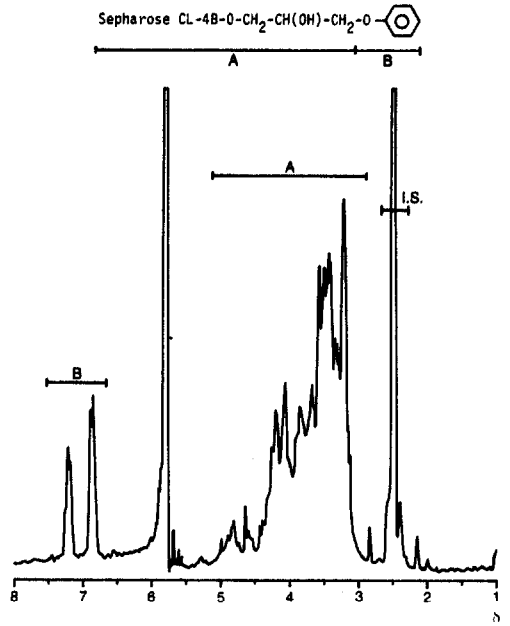

Fig. 1. ^1H NMR spectrum of partially hydrolysed Phenyl-Sepharose CL-4B in DMSO-d_6. The small amount of DMSO containing ^1H was used as internal standard (I.S.).

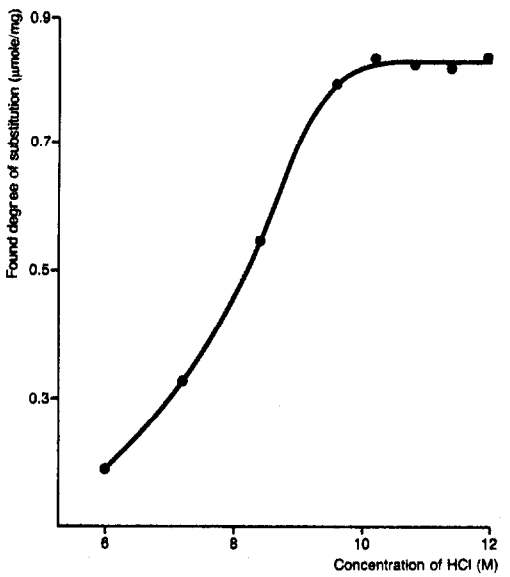

Fig. 2. Dependence of the spectrophotometrically found ligand content in Phenyl-Sepharose CL-4B on the concentration of hydrochloric acid used in the hydrolysis step.

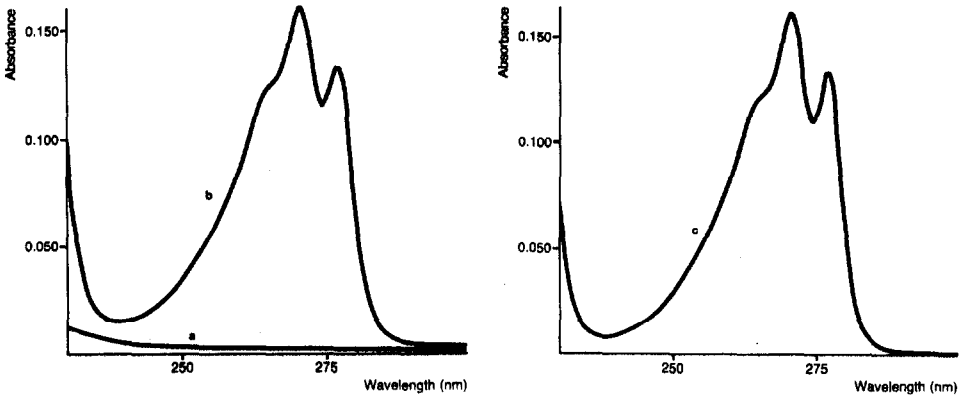

Fig. 3. UV spectra of hydrolysed Sepharose CL-4B (a) and Phenyl-Sepharose CL-4B (b) and of 0.10 m*M* 2-phenoxyethanol (c). For details see Experimental section.

atoms in the same ether. From this it follows that the degree of substitution (*S*) can be written as:

$$S = \frac{X \cdot 10^3}{150.18\ (100 + X)}\ \mu\text{mol/mg} \qquad (3)$$

RESULTS AND DISCUSSION

Ultraviolet spectrophotometry

UV-absorbing ligands covalently coupled to a gel matrix can be quantified spectrophotometrically if a solubilizing medium is chosen [14]. In this method we have used hydrochloric acid to cleave the glycosidic linkages in Sepharose CL-4B. More specifically, 10 *M* hydrochloric acid is required for the total dissolution of the dried gel in methanol (Fig. 2). A spectrum of hydrolysed Phenyl-Sepharose CL-4B is depicted in Fig. 3, which also shows that the interference from the gel matrix is low.

TABLE I

DEGREE OF SUBSTITUTION ON DIFFERENT BATCHES OF PHENYL-SEPHAROSE CL-4B DETERMINED BY THREE METHODS

Year of production	*Degree of substitution (μmol/mg dry gel)**		
	*Spectrophotometry***	*^1H NMR (spectroscopy****)	*Carbon analysis§*
1981	0.80 ± 0.05	0.89 ± 0.04	0.83 ± 0.11
1983	0.89 ± 0.03	0.93 ± 0.04	0.89 ± 0.22
1983	0.86 ± 0.05	0.88 ± 0.04	0.83 ± 0.22

* Values reported with a confidence interval of 95%.
** Pooled S.D. = 0.033 (degrees of freedom = 42).
*** Pooled S.D. = 0.017 (degrees of freedom = 3).
§ Pooled S.D. = 0.14 (degrees of freedom = 9).

TABLE II

SPECTROPHOTOMETRIC DETERMINATION OF THE LIGAND ON DIFFERENT BATCHES OF PHENYL-SEPHAROSE CL-4B

Year of production	*Batch No.*	*Degree of substitution (μmol/mg dry gel)**	*Year of production*	*Batch No.*	*Degree of substitution (μmol/mg dry gel)**
1977	9705	0.93 ± 0.05	1983	28 567	0.83 ± 0.05
1979	8565	0.90 ± 0.05	1983	32 351	0.89 ± 0.03
1979	12 514	0.81 ± 0.05	1983	33 555	0.86 ± 0.03
1980	16 110	0.88 ± 0.03	1983	33 777	0.86 ± 0.05
1980	18 581	0.80 ± 0.05	1984	35 016	0.80 ± 0.02
1981	19 418	0.80 ± 0.05	1985	38 578	1.01 ± 0.05
1982	19 419	0.93 ± 0.03	1985	00 323	0.97 ± 0.02
1982	28 566	0.80 ± 0.05			

* Values reported with a confidence interval of 95% calculated with a pooled S.D. = 0.033 (degrees of freedom = 42).

Furthermore, the spectrum of bonded phenyl groups coupled to the gel with the corresponding glycidyl ether is very similar to that of free 2-phenoxyethanol (Fig. 3). The molar absorptivity (ε) of 2-phenoxyethanol was determined to $1.76 \cdot 10^3$ M^{-1} cm^{-1}. This value was used in the calculation of the degree of substitution. The results from fifteen different batches, produced between 1977 and 1985, are presented in Tables I and II.

^1H NMR spectroscopy

The hydrolysis procedure of Phenyl-Sepharose CL-4B, described above, solubilizes the gel in DMSO-d_6. The hydrolysed gel can be characterized as short polygalactanes having ether-linked substituents. A typical NMR spectrum of such a solution is shown in Fig. 1. For the determination of the amount of phenyl groups the NMR signal from the aromatic protons are integrated and summarized. The reliability of the standard addition method has been verified by showing that the calibration graph of phenol was linear and had an intercept at the origin. Furthermore, the pulse delay has been chosen so that the aromatic protons had time to relax towards their equilibrium value. The importance of the operating parameter pulse delay is illustrated by the fact that the relaxation times of the phenyl protons are shorter when the phenyl group is coupled to the gel (*ca.* 1.0 s) compare with free uncoupled phenol molecules (*ca.* 6.7 s). The reliability of the integrated absorption signals has further been confirmed by showing that unsubstituted Sepharose CL-4B does not interfere with the evaluation of the peak areas.

The degree of substitution was determined on three different batches (Table I).

Carbon analysis

The degree of substitution was determined by carbon analysis on three batches of Phenyl-Sepharose CL-4B (Table I). The greater uncertainty of this method com-

pared with the two others can be deduced from the fact that the carbon contents in unsubstituted and substituted Sepharose CL-4B are of the same magnitude[10].

CONCLUSION

An important consideration in the selection of an analytical method is the accuracy and the precision of the result. Comparison of the phenyl content in Phenyl-Sepharose CL-4B determined by the three methods described shows that they consistently yield results showing only random differences at a 95% confidence level (Table I). Therefore it is concluded that no significant systematic errors are present. However, the higher precision of the NMR method and the UV method (Table I) makes these methods more reliable for quality control of the ligand content. The UV method is now used in the authors' laboratory, mainly because of the simpler instrumentation involved.

In this study the ligand density is determined on a dried gel. Compared with sampling of a certain settled gel volume this procedure is simpler and more reliable[10,15]. However, if a rough estimate of the density is wanted per millilitre of settled gel the dry weight of Phenyl-Sepharose CL-4B can be used (*ca.* 32 mg/ml). Moreover, from the molecular weight of phenyl glycidyl ether (150 g/mol) and anhydrodisaccharide unit of agarose (306 g/mol), it can easily be calculated that a ligand density of 0.90 μmol/mg dry gel means that one phenyl group is linked to a hexasaccharide unit. However, this calculation does not take account of the contribution from the cross-linker.

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