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Note

Quantitative determination of the ligand in Phenyl-Sepharose FF with proton nuclear magnetic resonance and derivative ultraviolet spectroscopy

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Phenyl-Sepharose FF, a derivative of the cross-linked agarose gel Sepharose FF, is a gel for large-scale hydrophobic interaction chromatography (HIC). The phenyl groups are coupled to the agarose gel via the reaction of phenyl glycidyl ether with Sepharose FF.

Quality control of the ligand content of different HIC gels demands simple and accurate methods with high precision[1]. UV-absorbing ligands covalently coupled to a gel matrix can easily be quantified spectrophotometrically if a solubilizing medium is chosen[2]. Recently, spectrophotometric methods for the determination of phenyl ligands in Phenyl-Sepharose CL-4B[3] and Phenyl-Superose[4] have been reported. These methods differ in the method of solubilizing the gel matrix. Phenyl-Sepharose CL-4B is hydrolysed by hydrochloric acid whereas Phenyl-Superose, which is cross-linked to a higher degree[5], requires boron tribromide[4]. The chemical stability of Phenyl-Sepharose FF is intermediate between those of these two gels, but hydrochloric acid can be used to solubilize the gel.

This paper describes the modifications of the UV method used for Phenyl-Sepharose CL-4B to suit the new support (Sepharose FF). It also describes the elimination of disturbing absorption bands from Sepharose FF by second-derivative UV absorption spectroscopy. For elucidation of systematic errors a ^1H NMR method was also applied.

EXPERIMENTAL

Chemicals and apparatus

Hydrochloric acid, acetone, methanol and phenoxyethanol were of analytical-reagent grade. Phenyl-Sepharose FF and Sepharose FF were obtained from Pharmacia (Uppsala, Sweden). Deuterium chloride and [$^2H_6$]dimethyl sulphoxide (isotopic purity greater than 99.5%) were purchased from Ciba-Geigy (Basle, Switzerland).

A Shimadzu UV-240 spectrophotometer, equipped with a PR-1 graphic printer, OPI-2 option program/interface and matched 1-cm quartz cells, was used for the spectrophotometric measurements. The scanning speed was 50 nm/min. The second-derivative spectra were derivatives of stored data with slit width 1 nm and derivative wavelength difference 1 nm. The wavelength range was 250–300 nm.

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The ^1H NMR spectra were recorded with a Jeol FX 200 199.5-Hz instrument. In the pulsed NMR experiments the number of pulses was 100, the pulse time 7 μs, the pulse delay 15 s, the acquisition time 2 s and the delay between pulse and acquisition 50 μs. The spectral range explored was 2000 Hz.

Sample pre-treatment

About 2 ml of homogenized Sepharose 6 FF or Phenyl-Sepharose FF were transferred into a glass filter funnel (G-4), washed with water, shrunk with acetone and finally dried at 70°C for 15 h and stored in a desiccator.

Determination of the ligand content by second-derivative UV spectroscopy

A 20-mg amount of the dried gel was hydrolysed at 20°C with 2.0 ml of concentrated hydrochloric acid for 15 min and the hydrolysed gel was diluted to 10.0 ml with methanol. An aliquot of this solution was further diluted 10-fold before the second-derivative spectrum between 250 and 300 nm was registered. The absorbance was evaluated according to Fig. 2.

A calibration graph for phenoxyethanol in solutions of hydrolysed unsubstituted gel matrix was constructed for the concentration range 0.05–0.2 m*M*.

Determination of the ligand content by ^1H NMR spectroscopy

The dried gel (20 mg) was hydrolysed with 200 μl of concentrated deuterium chloride at 70°C for 45 s and then cooled in an ice-bath. The sample was diluted with 1.00 ml of [$^2H_6$]dimethyl sulphoxide and a ^1H NMR spectrum was registered for the solution. The peaks from the isotopic impurities in [$^2H_6$]dimethyl sulphoxide served as internal standard. Standard solutions of phenoxyethanol in hydrolysed unsubstituted Sepharose 6 FF in the concentration range 5–12 m*M* were registered in the same way.

RESULTS AND DISCUSSION

Zero-order and second-derivative UV spectroscopy

Fig. 1 shows the absorption spectra of hydrolysed Phenyl-Sepharose FF and unsubstituted agarose matrix (Sepharose FF). It also shows the interferences from Sepharose FF on the spectrum of the phenyl groups. The lesser cross-linked gel Sepharose CL-4B does not give this type of interference (see Fig. 3 in ref. 3). Moreover, the UV response of hydrolysed Sepharose FF varies from one gel sample to another. Finally, the calibration graph for phenoxyethanol based on the measurement of the absorbance at 273 nm minus a blank value at that wavelength gives an intercept above the origin.

The observed interferences are probably caused by residues of large polysaccharide units of the gel matrix which result in light scattering. It can be seen that longer hydrolysis times do not decrease the absorbance contribution from Sepharose FF. As the background UV signal varies slowly with wavelength (Fig. 1), it should be possible to eliminate this interference by registering the second-derivative spectrum instead of the zero-order spectrum[6,7].

The second-derivative spectra of hydrolysed Phenyl-Sepharose FF and Sepharose FF are depicted in Fig. 2 which shows that the interference from the agarose

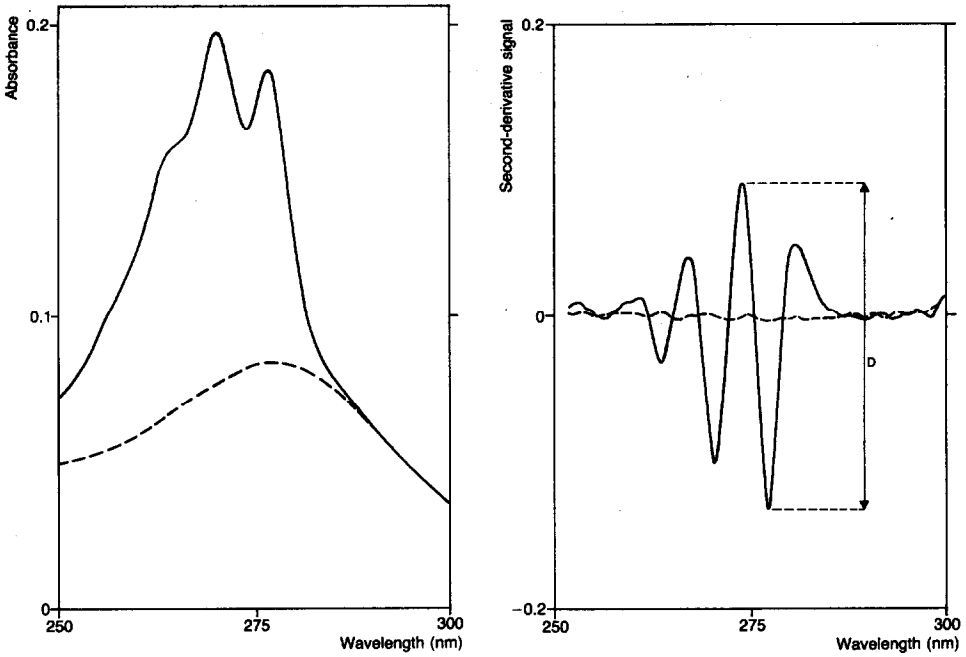

Fig. 1. UV spectra of hydrolysed Phenyl-Sepharose FF (solid line) and Sepharose FF (broken line).

Fig. 2. Second-derivative UV spectra from Fig. 1. Solid line, hydrolysed Phenyl-Sepharose FF; broken line, hydrolysed Sepharose FF.

matrix is removed. Phenoxyethanol has been shown to have a spectrum very similar to that of the bonded phenyl groups coupled to the gel[3] and was used as calibration standard also in this study. The equation of the calibration graph is

$$y = 3.71x + 2.90 \cdot 10^{-4} \quad (1)$$

where y is the absorbance D in Fig. 2 and x the concentration of phenoxyethanol in mM. Eqn. 1 indicates that the systematic error observed for the calibration graph in the zero-order UV method is eliminated. The correlation coefficient of the calibration graph calculated by linear regression was 0.999 over the investigated concentration range.

A series of six different development batches were analysed by second-derivative UV absorbance spectroscopy and the results are presented in Table I. The pooled standard deviation (s) was estimated to be 0.01 μmol/mg dry gel where s had 15 degrees of freedom.

^1H NMR spectroscopy

In order to study systematic errors, a ^{1}H NMR method was also applied to the determination of the ligand content. The NMR method used in this study was the same as that developed for Phenyl-Sepharose CL-4B[3] except that Phenyl-Sepharose FF was hydroysed in concentrated instead of 6.3 M deuterium chloride and

TABLE I

DEGREE OF SUBSTITUTION ON DIFFERENT DEVELOPMENT BATCHES OF PHENYL-SEPHAROSE FF BY TWO INDEPENDENT METHODS

Degree of substitution (μmol/mg dry gel)*	
Second-derivative UV spectroscopy	*^1H NMR spectroscopy*
0.45 ± 0.02	0.43 ± 0.03
0.32 ± 0.01	0.29 ± 0.03
0.32 ± 0.01	0.31 ± 0.02
0.25 ± 0.02	–**
0.16 ± 0.02	–**
0.64 ± 0.02	0.62 ± 0.03

* Values reported with a confidence interval of t = 95%; see text for details.
** Not analysed.

that phenol was substituted for phenoxyethanol as a calibration standard. The calibration graph for phenoxyethanol was linear and had an intercept at the origin. The pulse delay (15 s) was chosen so that the aromatic protons had time to relax towards their equilibrium value. The importance of this has been discussed earlier[1,3].

The results from four different development batches are presented in Table I. A pooled standard deviation (s) of 0.02 μmol/mg dry gel was achieved where s had 5 degrees of freedom.

CONCLUSION

This study has demonstrated the use of second-derivative UV absorption spectroscopy for the determination of the ligand content in hydrolysed Phenyl-Sepharose FF. Interferences due to broad-band matrix absorption are eliminated. Comparison of the phenyl content of Phenyl-Sepharose FF determined by the two methods described here shows that the methods consistently yield the same results at a confidence level of 95%. Therefore, it is concluded that no significant systematic errors are present.

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