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THREE INDEPENDENT METHODS FOR QUANTITATIVE DETERMINATION OF OCTYL COVALENTLY COUPLED TO SEPHAROSE CL-4B

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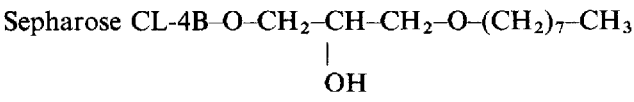
SUMMARY

Quantification of the octyl content in Octyl-Sepharose CL-4B was accomplished by three independent methods. Firstly, the ^{1}H NMR spectrum was registered on a deuterium chloride hydrolysed gel. Secondly, gas chromatography was applied to the ether-linked ligands cleaved by boron tribromide. Finally, the gel was combusted to carbon dioxide and elemental carbon analysis was performed. The results from the three methods indicate only random errors at a confidence level of 95%. All developed methods are therefore usable for the determination of the ligand content.

INTRODUCTION

Octyl-Sepharose CL-4B gel is frequently used for hydrophobic interaction chromatography of proteins and peptides[1–4]. The gel is synthesized by the reaction of octyl glycidyl ether with Sepharose CL-4B to give the partial structure:



The accurate determination of the amount of coupled octyl is highly important. The quality control of each production batch demands precise analytical methods as does the proof of ligand leakage from the support. When investigating the effect of ligand coverage on separation accurate determinations are also required.

A gas chromatographic (GC) method for the quantitative determination of octyl on Octyl-Sepharose CL-4B based on the ether cleavage by boron tribromide has recently been presented[5]. In order to adapt this method to a dried gel and to optimize and simplify the cleavage procedure this method has now been reinvestigated.

For comparison a ^{1}H NMR method has been developed in which the degree of substitution is evaluated by the standard addition of octanol to a solubilized Octyl-Sepharose CL-4B. Earlier, a ^{1}H NMR method for the determination of the ligand

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content was described[6]. However, it suffers from some severe drawbacks, exemplified by the requirement of precise knowledge of the gel matrix for correct interpretation.

A third independent method, which is based on the determination of the carbon content of the substituted and unsubstituted Sepharose CL-4B, has also been developed. This method supports the reliability of the two others.

EXPERIMENTAL

Chemicals and apparatus

2-Bromooctane, 4-bromooctane, boron tribromide, and octyl-β-D-glucopyranoside were of purum quality. Dichloromethane, methanol, dodecane, octanol and 1-bromooctane were of p.a. quality. Deuterium chloride and [$^2H_6$]dimethylsulphoxide (isotopic purity >99.5%) were from Ciba-Geigy (Basle, Switzerland). Octyl-Sepharose CL-4B and Sepharose CL-4B were obtained from Pharmacia (Uppsala, Sweden).

An HP 5790 gas chromatograph equipped with a flame ionization detector was used. The samples (1 μl) were injected onto a glass column (2 m × 4 mm I.D.) packed with 10% Apiezon L on Chromosorb W HP (80–100 mesh). The carrier gas flow-rate was 30 ml/min, the oven temperature 145°C, and the injector and detector temperatures 180°C and 200°C, respectively.

The NMR spectra were recorded with a Jeol FX 200 instrument at 199.5 Hz. In the pulsed NMR experiments the number of pulses was 100, the pulse time 7 μsec, the pulse delay 20 sec, the acquisition time 2 sec, and the delay between pulse and acquisition was 50 μsec. The spectral range explored was 2000 Hz.

Determination of the degree of substitution by gas chromatography

Preparation of the gel. About 10 ml of Octyl-Sepharose CL-4B were transferred to a glass filter funnel (G-4), washed with water, shrunken with acetone and finally dried at 105°C for 15 h. The dry gel was then kept in an desiccator.

Cleavage. About 40 mg of the dry gel were placed in a 10-ml measuring flask together with 500 μl of dichloromethane and kept at 25°C. The reaction was started with 800 μl of a solution of boron tribromide (1.38 mmol) in dichloromethane, which was freshly prepared every week, protected from moisture and stored at −30°C. After a reaction time of 30 min, unchanged boron tribromide was destroyed by hydrolysis with 800 μl of a 10% sodium hydroxide solution in water, and 20 μmol of *n*-dodecane were added as internal standard followed by dilution in methanol to volume.

The cleavage of a model substance, octyl-β-D-glucopyranoside, was performed in the same way.

For determination of the cleavage products standard solutions of 4-bromooctane, 2-bromooctane, 1-bromooctane and octanol in methanol were prepared in the concentration range 0.1–5 m*M*. All solutions were 2 m*M* in dodecane.

Determination of the degree of substitution by ^1H NMR spectroscopy

The gel was prepared as above, and 20 mg of the dried gel were hydrolysed with 200 μl of 38% deuterium chloride at 70°C for 35 sec. The hydrolysed gel was cooled in an ice-bath for 1 min, after which 2.00 ml of [$^2H_6$]dimethylsulphoxide

(DMSO-d_6) were added. From this mixture two samples of 1.00 ml each were taken. To one of the samples 100 μl of DMSO-d_6 were added and to the other 100 μl of DMSO-d_6 containing 10 μmol of octanol. NMR spectra were registered on these solutions. Standard solutions of octanol in DMSO-d_6 in the concentration range 2–20 mM were registered in the same way. The peaks from the H_A, H_B and H_C protons were integrated. The internal standard was isotopic impurities of DMSO-d_6 (Fig. 1).

The relaxation time for octanol and the isotopic impurities of DMSO-d_6 were measured in different surroundings by the inversion recovery method[7].

Determination of the degree of substitution by carbon analysis

Octyl-Sepharose CL-4B and the underivatized gel (Sepharose CL-4B) used as basic material for the substituted gel were washed and dried as above. The content of carbon was then determined on both gels.

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

$$Z = \frac{100\left(Y + \frac{132.12}{186.30}X\right)}{(100 + X)} \tag{1}$$

Rearrangement of this equation yields.

$$X = \frac{100\,(Z - Y)}{(70.92 - Z)} \tag{2}$$

where Y and Z are the degrees of carbon content in weight per cent of Sepharose CL-4B and Octyl-Sepharose CL-4B, respectively. The molecular weight of octyl glycidyl ether is 186.30 g/mol and the weight of the carbon atoms in the same ether is 132.12 g/mol. The degree of substitution (S) in μmole/mg can be thus written as:

$$S = \frac{X \cdot 10^3}{186.30\,(100 + X)} \tag{3}$$

RESULTS AND DISCUSSION

Description of the methods and the sampling procedure

The three independent methods for the determination of the ligand content in Octyl-Sepharose CL-4B were developed to investigate and identify errors and to work out one accurate method. The methods were chosen to be chemically and physically disparate so that interfering factors would affect the results in different ways. In the NMR method only the glycoside bonds are cleaved, whereas the GC method is based on the disruption of the ligand from the matrix. The third method implies total combustion of Octyl-Sepharose CL-4B to carbon dioxide.

In the determination of ligand density, sampling of substituted agarose gels normally means that a certain settled gel volume is taken[5,8]. This sampling procedure

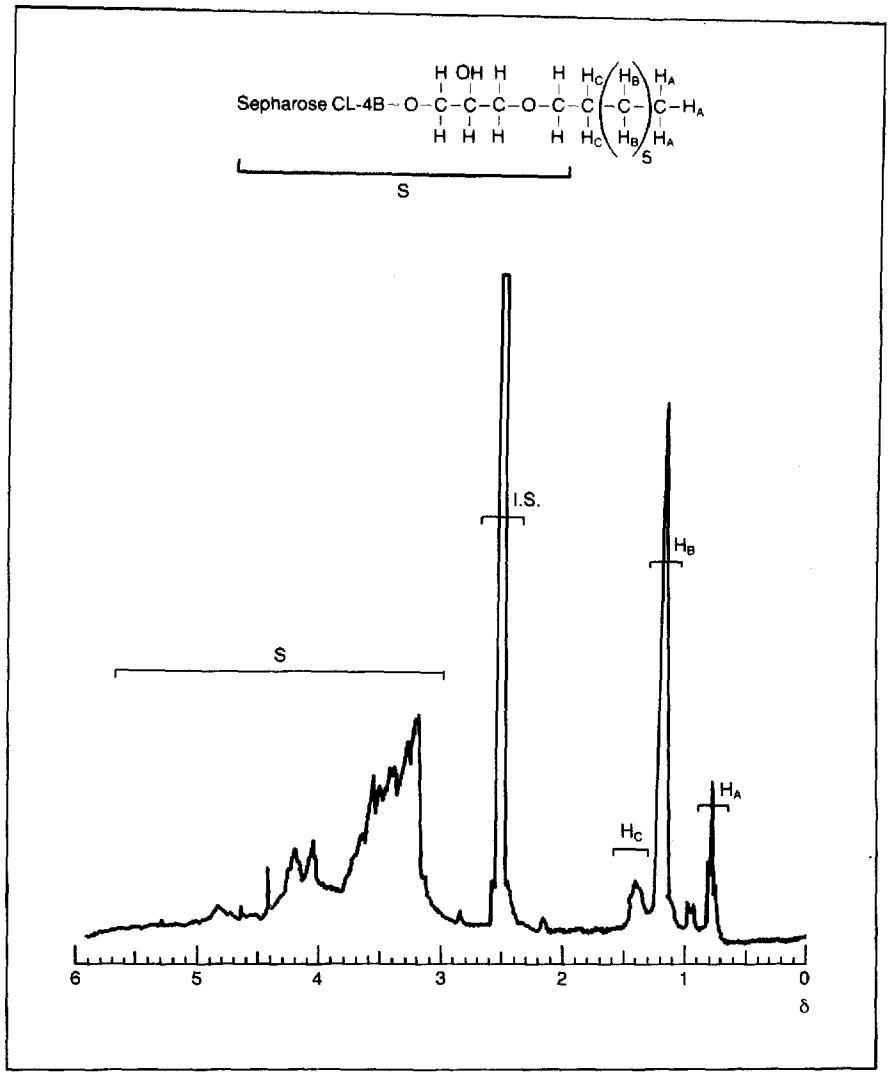

Fig. 1. NMR spectrum of partially hydrolysed Octyl-Sepharose CL-4B in DMSO-d_6. The small amount of DMSO-d_6 containing ^1H was used as internal standard.

is unreliable because the gel volume varies with the hydrostatic pressure when the gel is settled, the choice of solvent and other parameters which influence the swelling of the gel (*e.g.* time for swelling, temperature, salt content)[9]. In this study these errors have been circumvented since the ligand density was analysed on a dried gel. The dry weight of Octyl-Sepharose CL-4B is *ca.* 40 mg/ml settled gel, which can be used if a rough estimation of the density is wanted per millilitre of settled gel.

NMR spectroscopy

The acidic hydrolysis of Octyl-Sepharose CL-4B as described above splits the agarose gel into shorter polygalactanes, which are soluble in DMSO-d_6. The NMR

TABLE I

RELAXATION TIMES OF THE INTERNAL STANDARD AND THE LIGAND IN DIFFERENT SURROUNDINGS

Compound	*Relaxation time (min)*	
	CH_2 protons	*CH_3 protons*
DMSO-d_6 in the sample solution*		7.5**
Octanol dissolved in DMSO-d_6	1.5	2.6
Octanol dissolved in hydrolysed Sepharose CL-4B	0.9	1.9
Octyl in the sample solution*	0.5***	1.5§

* See Experimental for details.
** Internal standard in Fig. 1.
*** H_B and H_C in Fig. 1.
§ H_A in Fig. 1.

spectrum of such a solution is shown in Fig. 1. For determination of the amount of octyl groups the peaks from the H_A, H_B and H_C protons are integrated and summed. However, this integral includes a small peak at $\delta = 1$, which emanates from the gel matrix (Fig. 1). The magnitude of this peak is *ca.* 5% of the total integral and is therefore subtracted during the calculation of the ligand content.

Since the relaxation time of free octanol differs from that of octyl groups bonded to galactose residues (Table I), it is important to use a pulse delay long enough to allow all the octyl groups to relax. The value of pulse delay chosen here (20 sec) also allows the DMSO impurities used as internal standard to relax towards their equilibrium value (Table I).

The calibration graph of octanol was found to be linear with an intercept at origo.

The degree of substitution was determined by this NMR method for five dif-

TABLE II

QUANTITATIVE DETERMINATION OF THE LIGAND IN DIFFERENT BATCHES OF OCTYL-SEPHAROSE CL-4B WITH THREE INDEPENDENT METHODS

Year of production	*Batch No.*	*Degree of substitution* (μmol/mg dry gel)*		
		Gas chromatography	*NMR spectroscopy*	*Carbon analysis*
1977	3375	1.07 ± 0.06		
1977	9080	0.69 ± 0.06	0.69 ± 0.08	
1979	11143	0.80 ± 0.06		
1981	18580	0.90 ± 0.06	0.87 ± 0.07	1.01 ± 0.07
1982	24768	0.99 ± 0.06		
1983	28565	0.84 ± 0.06	0.77 ± 0.08	
1983	32349	1.01 ± 0.06	1.01 ± 0.08	1.00 ± 0.07
1984	33653	1.12 ± 0.06	1.11 ± 0.07	1.11 ± 0.07

* Values reported with a confidence interval of $t = 95\%$; see text for details.

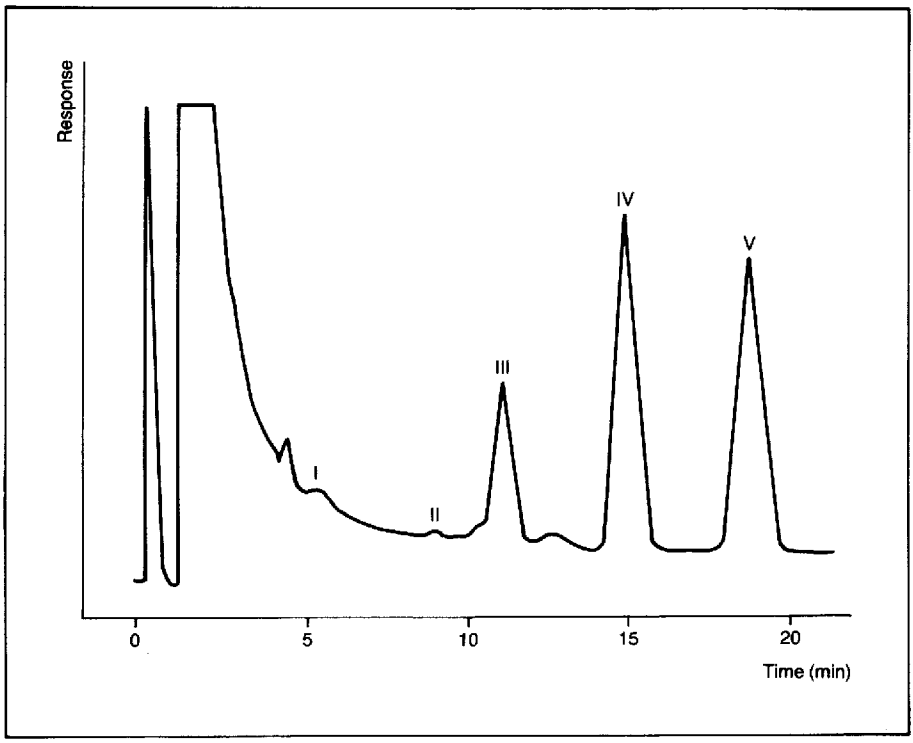

Fig. 2. Gas chromatogram of the cleavage of 40 mg of dry Octyl-Sepharose CL-4B using 1.38 mmol boron tribromide. Peaks: I = 4-bromooctane; II = octanol; III = 2-bromooctane; IV = 1-bromooctane; V = dodecane.

ferent batches of Octyl-Sepharose CL-4B (Table II) and gave a pooled standard deviation of 0.05 (n = 12).

Gas chromatography

Cleavage products. When octyl-β-D-glucopyranoside is cleaved by boron tribromide the products are octanol, 2-bromooctane, 1-bromooctane and small amounts of 4-bromooctane, as confirmed by comparison with the retention time of standards.

The cleavage pattern is the same when Octyl-Sepharose is cleaved (Fig. 2), but the proportions of the products may differ when the cleavage time or the amount of boron tribromide is changed (Figs. 3 and 4). However, the minor product 4-bromooctane is always less than 1% of the octyl-containing products.

Cleavage conditions. Complete cleavage of 25 μmol of octyl-β-D-glucopyranoside is achieved when the amount of boron tribromide is greater than 400 μmol (Fig. 3). This corresponds to an eight-fold excess of boron tribromide over reactive groups in the octyl-β-D-glucopyranoside.

To obtain quantitative cleavage when 25 μmol of octyl-β-D-glucopyranoside are cleaved together with 40 mg of dry Sepharose CL-4B, the amount of boron tribromide has to be increased to a ten-fold excess (500 μmol), and for quantitative

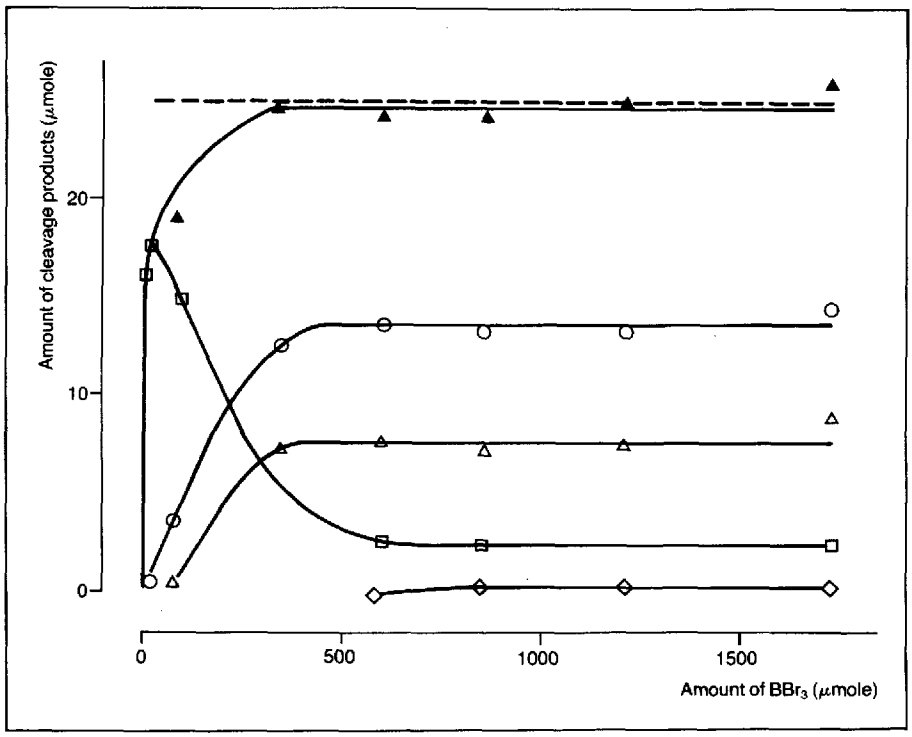

Fig. 3. Yield of products from the cleavage of 25 μmol of octyl-β-D-glucopyranoside by different amounts of boron tribromide after a cleavage time of 30 min. ◇ = Amount of 4-bromooctane; □ = amount of octanol; △ = amount of 2-bromooctane; ○ = amount of 1-bromooctane; ▲ = total amount of cleavage products. The dashed line represents 100% recovery.

cleavage of 40 mg of Octyl-Sepharose CL-4B 1000 μmol of tribromide were needed (Fig. 4). This consumption of boron tribromide of the agarose matrix has been pointed out previously[5,10].

As depicted in Fig. 5, a time of 30 min is enough to achieve total cleavage of Octyl-Sepharose CL-4B or octyl-β-D-glucopyranoside.

Estimation of the degree of substitution. The amount of octyl groups coupled to Sepharose CL-4B was evaluated from the amounts of octanol, 2-bromooctane and 1-bromooctane produced. These compounds give linear correlations between the concentration and the ratio of the area of the sample peak to that of the internal standard. All calibration graphs have intercepts at origo. A series of eight different batches of Octyl-Sepharose CL-4B has been investigated (Table II). These tests were performed by three different people and a pooled standard deviation of 0.06 (n = 32) was obtained.

Carbon analysis

The degree of substitution was determined by carbon analysis on three batches of Octyl-Sepharose CL-4B (Table II). A pooled standard deviation of 0.03 (n = 6) was obtained.

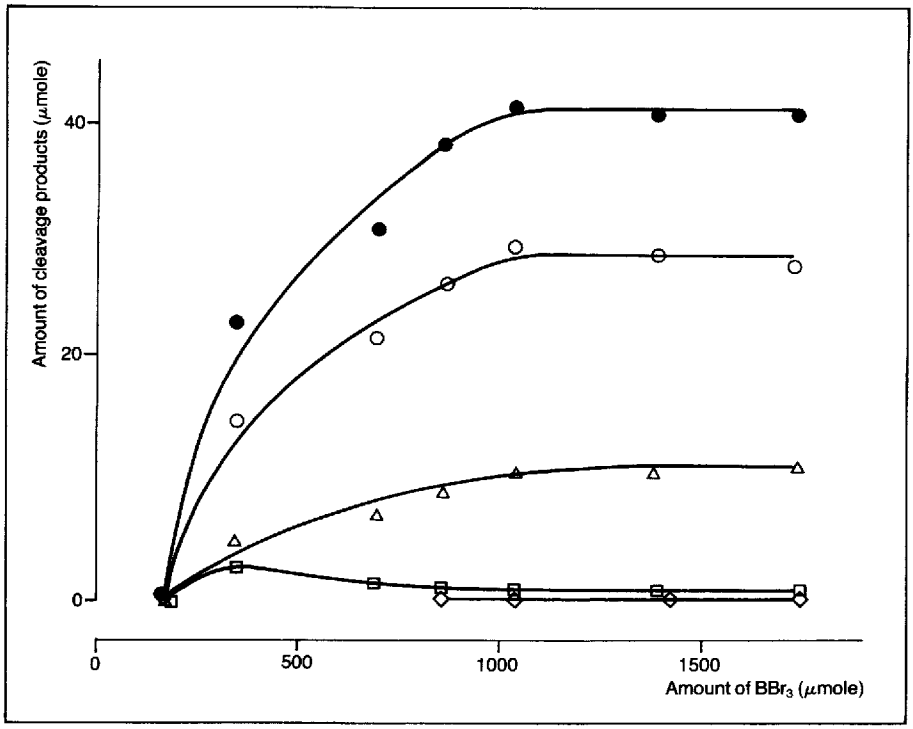

Fig. 4. Yield of products from the cleavage of 40 mg of dry Octyl-Sepharose CL-4B batch No. 32349 by different amounts of boron tribromide after a cleavage time of 30 min. ◇ = Amount of 4-bromooctane; □ = amount of octanol; △ = amount of 2-bromooctane; ○ = amount of 1-bromooctane; ● = total amount of cleavage products.

CONCLUSIONS

Comparison of the ligand densities determined by the three methods show that they consistently yield results showing only random differences at 95% confidence level (Table II). Therefore it is a reasonable assumption that no significant systematic errors are present. All three methods are consequently usable for the determination of the octyl content in Octyl-Sepharose CL-4B.

The uncertainty in the carbon analysis (± 0.05% C) and the fact that the carbon contents in unsubstituted and substituted Sepharose CL-4B are of the same magnitude makes this method unreliable at low ligand contents. Elemental analysis is therefore normally used where the immobilized ligand contains an element not represented in the gel matrix[11]. However, in many cases the carbon analysis is sufficiently accurate, especially if the carbon content in the ligand is high and the ligand is of high molecular weight.

One of the main limitations of the NMR method is the inherent lack of sensitivity. Therefore, the method of choice, if low ligand contents are to be analysed, is the GC method.

The GC method is now used in the authors' laboratory for quality control of

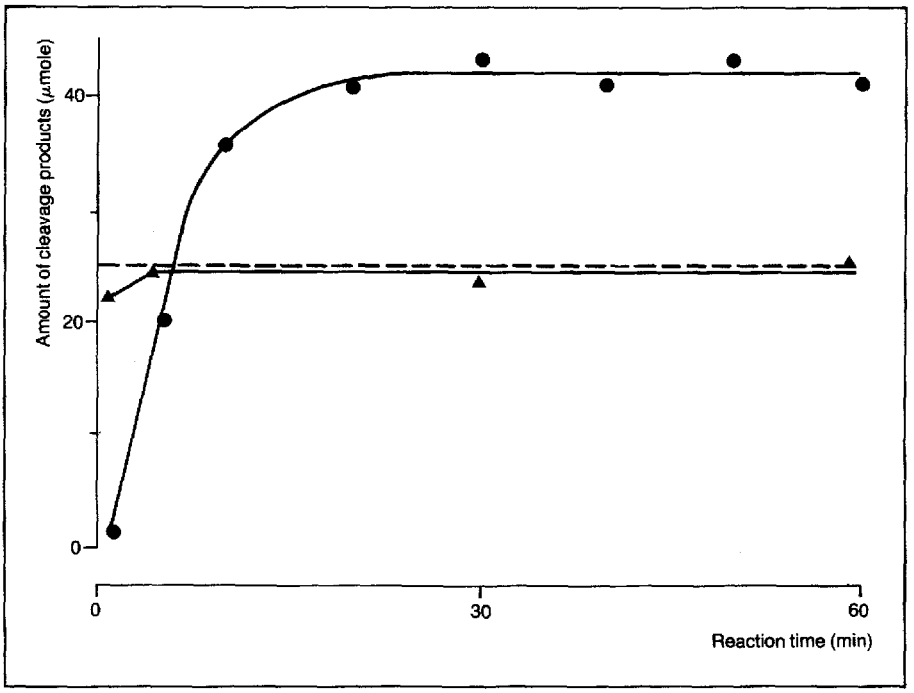

Fig. 5. The time dependence of the total yield of products from the cleavage of 25 μmol of octyl-β-D-glucopyranoside (▲) and 40 mg of dry Octyl-Sepharose CL-4B batch No. 32349 (●). The dashed line represents 100% recovery of the cleavage of 25 μmol of octyl-β-D-glucopyranoside.

the ligand content in Octyl-Sepharose CL-4B. This choice is based on the fact that the GC method also is used for other ligands[10].

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