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Note

Determination of polyethylene glycol bonded to epoxy-activated Sepharose 6B

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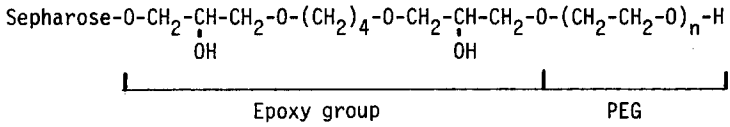
There is a need for analytical methods for the determination of ligands on affinity gels. Knowledge of the ligand concentration is required to optimize the ligand coupling reaction, to study column bleeding and when investigating the effect of ligand coverage on separation.

Previously described methods are based mainly on potentiometric titration[1,2] and UV measurement[3] on a suspended gel solution, NMR measurement on a partly hydrolyzed gel[4] and estimation of the consumption of a specific reagent after a complete reaction with the ligand[5,6].

This paper describes a method for the determination of polyethylene glycol (PEG) coupled to Sepharose 6B through 1,4-bis(2,3-epoxypropoxy)butane. The partial structure is:



The method is based on quantitative cleavage of the ether bonds in the ligand and the subsequent gas chromatographic (GC) determination of the cleavage products.

The ether cleavage is accomplished by use of boron tribomide (BBr_3). The use of boron halides for ether cleavage is well documented[7,8]. Recently, BBr_3 has become the reagent of choice for such reactions because it reacts cleanly and quantitatively under mild conditions[9]. It has been successfully used for the determination of the ligands octyl and phenyl bonded via etherlinkages to Sepharose CL 4B[10].

Column packings consisting of chemically bonded PEG on Sepharose 6B have been used to separate human platelets, granulocytes, lymphocytes and erythrocytes[11]. Also PEG-substituted Sephadex has been used in hydrophobic interaction chromatography[12]. Sepharose 6B substituted with PEG and a protein binding ligand has recently been used in aqueous two-phase systems for extraction of enzymes and proteins[13]. In this application PEG was bonded to the gel in order to distribute the adsorbent in the upper phase.

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EXPERIMENTAL

Chemicals

1,2-Dibromoethane, 2-bromo-1-ethanol, 1,4-dibromobutane and boron tribromide were of purum quality. Bromobenzene, PEG 20000, methanol and dichloromethane were of p.a. quality. Sepharose 6B and epoxy-activated Sepharose 6B were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

Apparatus

A HP 5790 gas chromatograph equipped with a flame ionization detector and a HP 3390 A integrator was used. The samples (1 μl) were injected onto a glass column (2 m × 2 mm I.D.) packed with 10% Apiezon L and 10% diethyleneglycol succinate on Chromosorb W AW DMCS (80–100 mesh). The carrier gas (nitrogen) flow-rate was 30 ml/min, the oven temperature 90°C and the injector and detector temperatures 120 and 150°C, respectively.

Determination of 0–6 mg PEG 20000

PEG-substituted Sepharose 6B (5 ml) was washed ten times with 5-ml portions of water. The gel was then centrifuged at 250 *g* for 10 min. One gram of the centrifugate was transferred to a glass filter-funnel and washed into the dichloromethane phase as described[14].

For cleavage, the water-free gel was placed in a 10-ml measuring flask together with 500 μl of dichloromethane and kept in a water-bath at 30°C. The reaction was started with 700 μl of a solution of boron tribromide (1.21 mmol) in dichloromethane which was freshly prepared every week, protected from moisture and stored at −30°C when not used. After a reaction time of 15 min, unreacted boron tribromide was destroyed by hydrolysis with a 10% sodium hydroxide solution (5 × 100 μl). As internal standard, 6.40 μmole of bromobenzene in methanol were added followed by dilution in methanol to volume.

When free PEG 20000 was cleaved, a known amount of the substance was placed in a 10-ml flask and treated in the same way as the washed gel suspension.

For determination of the cleavage products, standard solutions of 2-bromo-1-ethanol and 1,2-dibromoethane in methanol were prepared in the concentration range 0.05–1 mg/ml. All solutions were 0.640 m*M* in bromobenzene.

RESULTS

Cleavage products and recovery

When PEG is cleaved by boron tribromide the resulting products are 2-bromoethanol and 1,2-dibromoethane as confirmed by comparison with the retention times of standards. These compounds give linear correlations between the concentration and the ratio of the area of the sample peak to that of the internal standard. Both calibration graphs have an intercept at origo. When a solution of free PEG 20000 is treated with boron tribromide for more than 10 min, quantitative cleavage is obtained as estimated from the calibration graphs (Fig. 1). Quantitative cleavage also occurs for free PEG 20000 when added to a gel suspension of PEG-substituted epoxy-activated Sepharose 6B.

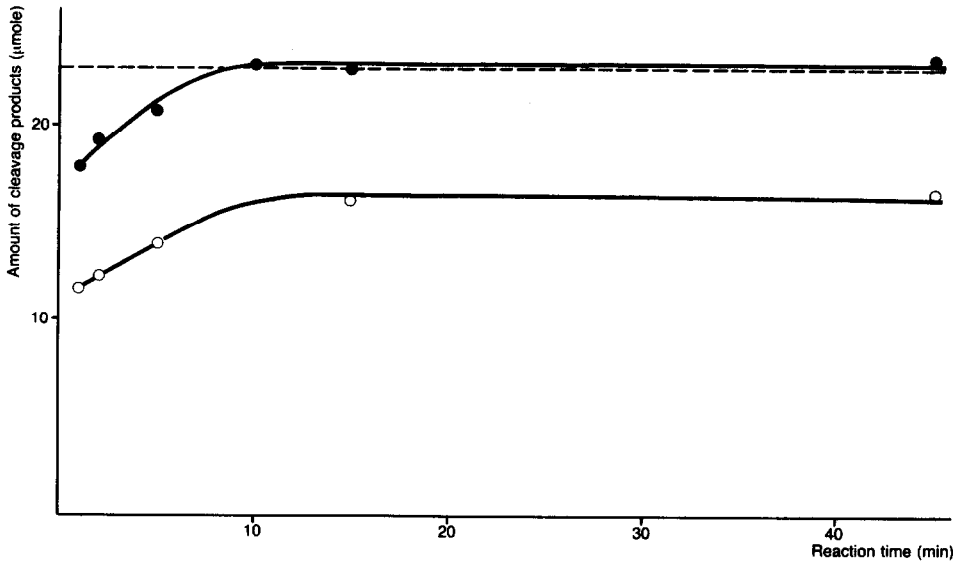

Fig. 1. The time dependence of the yield of the products, 2-bromoethanol and 1,2-dibromethane, upon cleavage of 1.00 mg PEG 20000 (●) and 1.00 g PEG 20000-substituted epoxy-activated Sepharose 6B [degree of substitution 0.070% (w/w)] (○) with 1.00 mmol boron tribromide. The dashed line represents 100% recovery of the cleavage products from 1.00 mg PEG 20000.

The chromatogram of a cleaved PEG-substituted epoxy-activated Sepharose 6B shows peaks from 2-bromoethanol and 1,2-dibromoethane and a large peak at about 23 min (Fig. 2). Cleavage experiments with Sepharose 6B and only epoxy-activated Sepharose 6B demonstrate that this peak emanates from the epoxy groups. The peak from 1,4-dibromobutane coincides with this peak. Small peaks appear be-

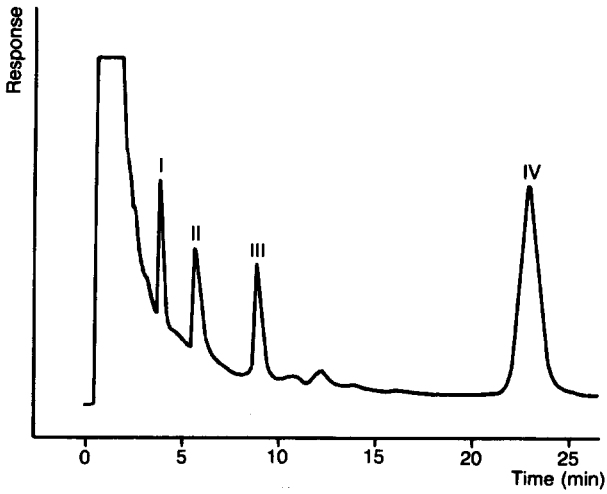

Fig. 2. Gas chromatogram of the cleavage products from 1 g PEG-substituted epoxy-activated Sepharose 6B (degree of substitution 0.2%) using 1.21 mmol boron tribromide. Peaks: I = 1,2-dibromoethane; II = 2-bromoethanol; III = bromobenzene; IV = 1,4-dibromobutane.

tween 10 and 13 min on the chromatogram (Fig. 2). These peaks originate from the Sepharose matrix and become very large unless the gels are washed free from water.

Cleavage conditions

A complete cleavage of 1 mg of free PEG 20000 is achieved when the amount of boron tribromide is higher than 100 μmol (Fig. 3). This corresponds to a four-fold excess of boron tribromide over the reactive ether groups in PEG. In order to obtain quantitative cleavage, the amount of boron tribromide must be increased to a nine-fold excess, when PEG 20000 is cleaved in a mixture with 1 g of PEG-bonded Sepharose 6B (Fig. 3). The cleavage pattern of PEG is the same regardless of the amount of boron tribomide, however the ratio of 1,2-dibromoethane to 2-bromoethanol increases when more than a fifty-fold excess of boron tribromide (1.5 mmol) is added (Fig. 3).

As depicted in Fig. 1, a reaction time of 15 min is enough to achieve total cleavage of gel-bonded or free PEG.

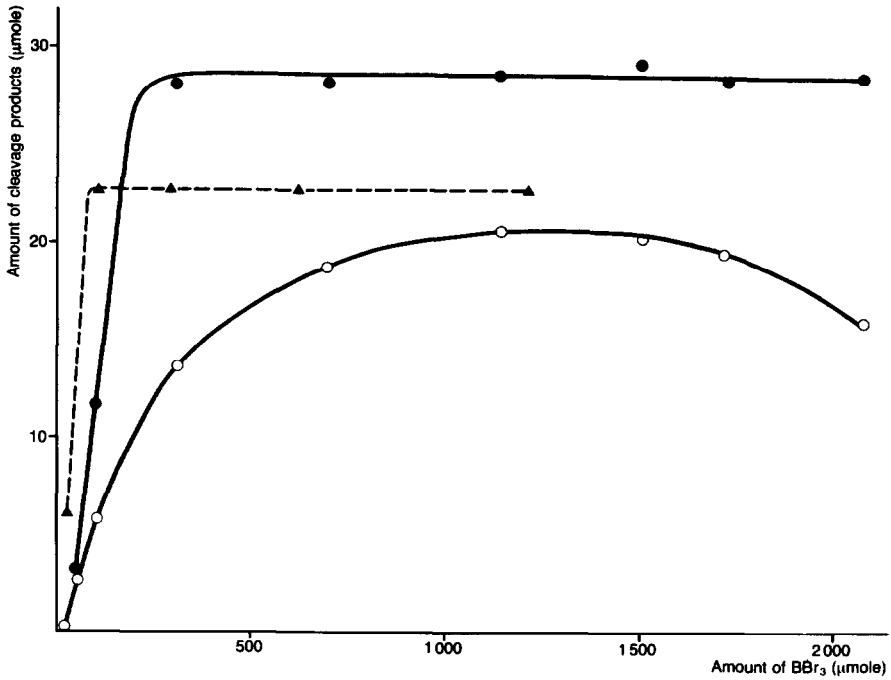

Fig. 3. Yield of 2-bromoethanol and 1,2-dibromoethane upon cleavage of 1.00 mg PEG 20000 (dashed line) and a mixture (full line) of 1.00 g PEG 20000-substituted epoxy-activated Sepharose 6B [degree of substitution 0.026% (w/w)] and 1.00 mg PEG 20000 by different amounts of BBr_3. ○, Amount of 2-bromoethanol; ▲ and ●, total amount of 2-bromoethanol and 1,2-dibromoethane.

Estimation of the degree of substitution

The amount of PEG bonded to epoxy-activated Sepharose 6B was evaluated from the amounts of 2-bromoethanol and 1,2-dibromoethane produced. To test the reproducibility, three separate determinations were performed. The results gave a

mean value of 1.23 mg PEG per g gel with a relative standard deviation of 2.0%. About twenty gels with degrees of substitution between 0.2 and 6.0 mg per g gel have been investigated with the boron tribromide method described above. From these experiments a relative standard deviation of less than 5% ($n = 2$) was obtained.

DISCUSSION

The presented method for cleavage and determination of gel-bonded PEG 20000 with boron tribromide is characterized by quantitative rupture of the ether linkages and an inherent sensitivity factor of about 400 since the cleavage products are 2-bromoethanol and 1,2-dibromoethane. These distinctive features make the method very sensitive and reliable even for the determination of small amounts of bonded PEG 20000. The method can of course also be applied to PEG having lower number-average molecular weights.

The usefulness of BBr_3 as ether cleavage reagent is further illustrated by the formation of 1,4-dibromobutane when epoxy-activated Sepharose 6B is cleaved. This cleavage of the epoxy groups provides the possibility to determine the total amount of these spacer groups. The investigation of the complete cleavage pattern of the epoxy groups is in progress.

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