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Note

Spectrofluorometric method for the quantitation of amino groups on solid supports

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Matrices containing primary amines bound at the extremity of a spacer arm are widely used for immobilizing ligands of biological interest in the fields of biospecific interaction and preparative affinity chromatography. The coupling of the ligand to the support is a delicate operation, often resulting in a low yield. It is thus necessary to determine the quantity of available amino groups on the support in order to optimize the reaction.

Currently, several methods are available for the quantitation of amino groups on supports. One of them[1] can be applied to all types of matrices, but it is relatively insensitive and requires large quantities of gel.

We propose a spectrofluorometric method based on the reaction of the amino groups on the support with an excess of OPA (*o*-phthalaldehyde) and assay of the excess of reagent with glycine[2]. The reaction is rapid, is carried out at room temperature, does not require solubilization of the support and consumes a small quantity of gel.

EXPERIMENTAL

Sepharose 4B and AH Sepharose 4B were obtained from Pharmacia (Uppsala, Sweden). Ultrogel AcA-34 and HMD Ultrogel AcA-34 were obtained from IBF (Villeneuve-La-Garenne, France). OPA, 2-mercaptoethanol, glycine, TNBS (2,4,6-trinitrobenzenesulphonic acid) and ethylenediamine were purchased from Sigma (St. Louis, MO, U.S.A.). In addition to ready-to-use aminated spacer arm gels, AE (aminoethyl) Sepharose 4B gels having various degrees of substitution were prepared by coupling with ethylenediamine on CNBr-activated Sepharose 4B as described elsewhere[3]. The first gel underwent 15 min of coupling, the second 1 h and the third two couplings of 1 h each.

The method of Antoni *et al.*[1] was used for the spectrophotometric assay of the amino groups. After the reaction of TNBS with the gel, its excess was assayed by determining the trinitrophenyl derivatives formed upon reaction with glycine. A di-

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rect determination of the trinitrophenyl derivatives of the agarose was also performed after dissolving the gel in a solution of hot 50% acetic acid in 3 *M* hydrochloric acid, as described by Failla and Santi[4].

Reaction of the gel with OPA and spectrofluorometric determination of the excess of OPA

Two freshly prepared reagents were used. For the reaction of the primary amines of the gel, 50 ml of reagent R1 were employed: 300 μl of 0.25 *M* OPA in ethanol and 300 μl of 4% 2-mercaptoethanol in 0.1 *M* potassium borate buffer, pH 9.5. For the determination of the excess of OPA, 60 ml of reagent R2: 1 ml of 0.06 *M* glycine and 1 ml of 0.5% 2-mercaptoethanol in borate buffer.

The gel (100 μl) was first incubated with 2 ml of reagent R1 for 5 min with stirring and was then centrifuged (at 1000 *g*). A 1-ml volume of the supernatant was diluted 1/40 with borate buffer and 2 ml of reagent R2 were added. After 1 min, a Jobin Yvon JY3 spectrofluorimeter equipped with a xenon light source was used at the excitation and emission wavelengths of 340 and 455 nm[5]. The quantity of primary amine in the sample was determined from a calibration curve prepared with known concentrations of OPA.

RESULTS AND DISCUSSION

The method proposed for the analysis of primary amino groups on supports requires the quantitation of the excess of OPA remaining after incubation with the support.

Several criteria guided the choice of support. OPA reacts with primary amines in the presence of 2-mercaptoethanol to form highly fluorescent thiosubstituted isoindole derivatives[2], but does not react directly with secondary amines, which require a prior reduction by sodium hypochlorite. This is important since the spacer arms contain a secondary amine at their point of attachment to the support. Also, OPA itself does not fluoresce and thus does not interfere with the determination. The reaction is very rapid and is carried out at room temperature on soluble or insoluble supports.

Under our experimental conditions, an excess of 2-mercaptoethanol or glycine does not destabilize the isoindole derivative. Glycine derivatives, on the other hand, are relatively unstable and so it is preferable to take the readings as early as 1 min. It is nevertheless possible to take readings after a longer period, *e.g.*, 30 min, providing this is done for all the determinations. We noted a decrease in fluorescence of 1.5% per min.

As a result of its destabilizing effect[6], OPA must not be present in too great an excess. The ideal quantity of OPA is about two to four times that of the amino groups.

In order to test our method, we examined various supports possessing variable quantities of amino groups, using several different methods (see Table I). The results obtained were entirely concordant. The advantages of our method, especially the excellent reproducibility, are summarized in Table II. Thus, repeated determinations on the same gels gave results that did not differ by more than 2%. In addition, its sensitivity enables highly variable quantities of amines to be determined by changing the volume of the gel or reagent used.

TABLE I

DETERMINATION OF AMINO GROUPS IN AGAROSE AND AGAROSE-POLYACRYLAMIDE GELS*: COMPARISON OF METHODS

Method	*Amino groups (μmol per ml packed gel)*				
	AH Sepharose 4B	*HMD Ultrogel AcA-34*	*AE Sepharose 4B***		
Present	8.1	8.2	4.5	8.9	14.7
Antoni *et al.*[1]	8.1	8.3	4.4	8.9	14.5
Failla and Santi[4]	7.9	–	4.3	8.9	14.6
Manufacturer***	6–10	6–10	–	–	–

* Experiments with the corresponding non-substituted gels showed no appreciable reaction under our experimental conditions.

** Sepharose derivatives obtained by coupling ethylenediamine on CNBr-activated Sepharose 4B.

*** Determination was by frontal analysis or potentiometrically[7].

The method is very simple to use and highly specific, sensitive, reproducible and does not require the dissolution of the support. It is carried out at room temperature and thus is independent of the thermal stability of the support, in contrast to other techniques[8–10] which are applicable only to insoluble matrices, *e.g.*, polystyrenes, silicas or porous glass, resistant to high reaction temperatures.

OPA reacts with the matrix in a non-denaturing aqueous medium and requires only a very small volume of gel.

The method proposed is thus well adapted to hydrophilic gels such as agaroses or polyacrylamides and in general appears applicable to all types of solid hydrophilic or hydrophobic supports. Finally, it may be useful and reliable for the quantitation of certain ligands immobilized on affinity chromatography supports[11].

TABLE II

COMPARISON OF DIFFERENT METHODS USED FOR THE DETERMINATION OF AMINO GROUPS ON AN HYDROPHILIC SUPPORT

	Method		
	Present	*Antoni et al.*[1]	*Failla and Santi*[4]
Reaction time	10 min	2.5 h	2.5 h
Incubation temperature	Ambient	37 and 25°C	37 and 50°C
Packed gel volume (ml)	0.1	0.5	0.5
Technical difficulties	Medium	Few	Few
Sensitivity	High	Low	Low
Reproducibility	High	Medium	Medium
Specificity	High	Medium	Medium
Restrictions	None	None	Insoluble gels

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