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A Spectrophotometric Assay for Solid Phase Primary Amino Groups

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ABSTRACT

A rapid, sensitive, and convenient spectrophotometric assay was developed for the measurement of amino groups on solid supports. This method is based on the reaction of amino groups of solids with an excess of *o*-phthaldialdehyde (OPA) and subsequent quantitative determination of unreacted OPA by reaction with glycine. Four solids possessing variable quantities of amino groups were examined. Results indicate that about 70% of the total surface amino concentration (determined by the microKjeldahl method) are available for ligand attachment. Unlike the spectrophotometric 2,4,6-trinitrobenzenesulfonic acid method, the OPA spectrophotometric assay is more rapid, sensitive, and convenient, and unlike the spectrofluorimetric OPA, it does not require sophisticated instrumentation.

Index Entries: Solid phase amino groups, assay of; *o*-phthaldialdehyde assay; spectrophotometric assay of solid phase amino groups.

INTRODUCTION

Solid supports containing primary amines bound at the extremity of spacer arms are widely used for immobilizing ligands of biological interest. In order to optimize the coupling of the ligand to the matrix, it is impor-

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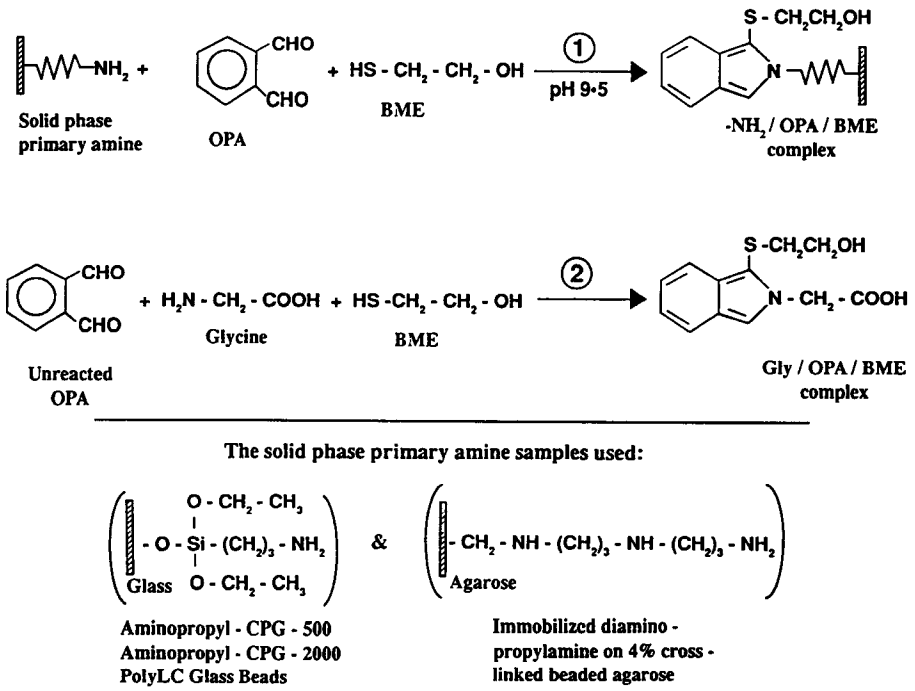

Fig. 1. Reaction of solid phase primary amino group with excess *o*-phthaldialdehyde (OPA) in the presence of β-mercaptoethanol (BME) ① and subsequent reaction of unreacted OPA with glycine and BME ② to form a Gly/OPA/BME complex that absorbs strongly at 340 nm.

tant to determine the quantity of available amino groups. Current methods that do not require solubilization of the matrix are the spectrophotometric 2,4,6-trinitrobenzenesulfonic acid (TNBS) method (*1*) and the spectrofluorimetric *o*-phthaldialdehyde (OPA) method (*2*). The former is time consuming and the latter requires sophisticated instrumentation.

The assay method proposed is based on the reaction of OPA and β-mercaptoethanol (BME) with primary amines. Roth (*3*) was the first to describe this method for the determination of amino acids. Applications of OPA have centered around use of fluorimetric methods for the determination of primary amines and peptides (*4–6*). It has also been reported that OPA adducts absorb strongly at 340 nm (*7–9*). Subsequently, spectrophotometric determinations of amino acids and of proteolysis in milk using an OPA assay have been reported (*10,11*).

We have developed a spectrophotometric OPA assay for the quantitation of amino groups in solids. It is based on the reaction of the amino groups in the solid with an excess of OPA and assay of the excess reagent with glycine. The reactions involved are shown in Fig. 1.

MATERIALS AND METHODS

Materials

The samples used in this study for the determination of amino groups in solid supports were aminopropyl (CPG-500) beads, aminopropyl (CPG-2000) beads, aminopropyl (PolyLC, nonporous) beads, and Sepharose-CL-4B diaminopropylamine. The first three samples were prepared by aqueous silanization (*12*). The cleaned porous and nonporous beads were reacted with 10% v/v aqueous solution of 3-aminopropyltriethoxysilane, pH 4, for 3 h at 70°C. The immobilized diaminopropylamine on cross-linked beaded agarose was obtained from Pierce (Rockford, IL).

Methods

For the determination of the total amino concentration on the solid support, a microKjeldahl-microdiffusion technique was employed (*13*).

The method of Antoni et al. (*1*) was used for the spectrophotometric TNBS assay of amino groups. After reaction of the TNBS with the solid support, its excess was assayed by determining the trinitrophenyl derivatives formed upon reaction with glycine.

Reaction of the Solid Support with OPA and Spectrophotometric Determination of the Excess of OPA

REAGENTS

The reagents were prepared as described by Hoinard et al. (*2*). Two freshly prepared reagents were made as described below:

For reaction of primary amines of the solid, reagent R_1 was made by combining 300 μL of 0.25*M* OPA in ethanol and 300 μL of 4% β-mercaptoethanol in 0.1*M* sodium borate, pH 9.5 and diluting to a final volume of 50 mL with water.

For the determination of excess OPA, reagent R_2 was made by combining 500 μL of 0.06*M* glycine and 500 μL of 0.5% β-mercaptoethanol in 0.1*M* borate buffer, pH 9.5, and diluting to a final volume of 30 mL with water.

PROCEDURE

A volume of packed glass beads or gel containing not more than 1.5 μmol of amino groups was first incubated with 2 mL of degassed reagent R_1 for 5 min at ambient temperature. To 50 μL of supernatant in a 3-mL cuvet, 2 mL 0.1*M* sodium borate and 100 μL of reagent R_2 were added. The solution was mixed briefly by inversion, incubated for 2 min at ambient temperature, and the absorbance at 340 nm was measured. A refer-

Table 1
Determination of Amino Groups
in Porous and Nonporous Glass Beads and Agarose Gel

Method	Amino groups (μmol/mL packed glass beads or gel)[a]			
	CPG-500, porous	CPG-2000, porous	PolyLC glass beads, nonporous	Seph-CL-4B, diaminopropylamine
OPA (spectrophotometric)	47.63 (0.78)	10.56 (0.38)	3.74 (0.12)	13.73 (0.23)
TNBS	29.56 (0.07)	7.78 (0.03)	2.43 (0.12)	10.37 (0.07)
MicroKjeldahl	66.12 (0.76)	14.56 (0.64)	5.11 (0.55)	18.66 (0.05)[b]

[a]Average and standard error (in parentheses) for triplicate or quadruplicate (micro-Kjeldahl) determinations.
[b]This value was calculated by assuming that amino nitrogen represents one-third of the total nitrogen.

ence sample was prepared composed of 2 mL of reagent R_1 without the solid support from which 50 μl were taken, diluted with 2 mL 0.1*M* sodium borate and 100 μL of reagent R_2 were added.

A blank was prepared for each sample, composed of 50 μL supernatant (or 50 μL of reagent R_1 for the reference sample) and 2 mL of 0.1*M* sodium borate with 100 μL of water added in place of glycine. The absorbance of each sample was measured against its own blank at 340 nm.

The concentration of amino groups on the solid support was determined from the difference between the absorbances of reference and sample, using a molar absorption coefficient $\epsilon = 6000 M^{-1}$ cm^{-1} (*11*).

RESULTS AND DISCUSSION

The method developed for analysis of primary amino groups in solids requires quantitation of excess OPA remaining after incubation with the matrix. Primary amines react with OPA and β-mercaptoethanol to form an adduct that absorbs strongly at 340 nm. The absorptivity ($\epsilon = 6000 M^{-1}$ cm^{-1}) was found to be similar for most α-amino groups (*11*). OPA is known to have a destabilizing effect on the $-NH_2$/OPA/BME complex (*2*) so it must not be present in large excess. An ideal quantity of OPA is about two to four times that of the amino groups. Data for analysis of primary amino groups in 3-aminopropyl derivatives of CPG-500, CPG-2000 and nonporous PolyLC glass beads and of diaminopropylamine Sepharose CL-4B are given in Table 1.

It is apparent from these data that values obtained by the spectrophotometric OPA method are closer to those determined by microKjeldahl than those measured by the TNBS assay. Not all of the amino groups present in the solid support react with either the OPA or TNBS reagent, most

likely owing to lack of accessibility. However, more than 70% of these groups are reactive with OPA, whereas only 50% react with TNBS.

Lack of chemical reactivity of all amino groups incorporated into a silica matrix surface by silanization with organosilanes is not unexpected. For example, only 40% of the surface sites were reactive with glycine methyl ester following succinylation of the amino groups of aminopropyl-glass and activation of carboxyl groups with excess carbodiimide (*12*).

Although the method was developed specifically to assay amino groups in glass, silica and agarose supports, there is no reason to expect that it would not be suitable for assay of amino groups in other solids. Thus, it may be possible to adapt this method for assay of amino groups in complex materials.

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