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Coupling Capacity of Solid-Phase Carboxyl Groups

Determination by a Colorimetric Procedure

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ABSTRACT

A simple colorimetric procedure for determining the coupling capacity of solid-supported carboxyl groups has been developed. The carboxyl groups of a solid support were coupled to cystamine at pH 4–4.5, using a water soluble carbodiimide 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiime as the condensing reagent. The solid-phase coupled disulfides were then reduced to sulfhydryl groups by treating the solid phase with dithiothreitol. For every one carboxyl group coupled with cystamine, one solid-phase sulfhydryl is introduced. After removing all of the reducing reagents by extensive washing, the sulfhydryl content, which is equivalent to the carboxyl groups of the gel, was quantified by using 5,5′-dithiobis-(2-nitrobenzoic acid), the Ellman's reagent.

Index Entries: Carboxyl groups, solid-phase, coupling capacity of; coupling capacity, of solid-phase carboxyl groups; colorimetric procedure, for determination of coupling capacity of solid-support carboxyl groups.

INTRODUCTION

The carboxyl groups of a solid support can be quantified by acid-base titration (*1,2*). This method, however, did not provide the coupling capacity of the solid-supported carboxyl groups. Cuatrecasas and Parikh (*3*)

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coupled ^{14}C-alanine to the *N*-hydroxysuccinimide (NHS) ester of the solid-phase carboxyl groups to determine the coupling capacity of the latter. Goldstein (*4*) developed a nonisotopic method that used dicyclohexylcarbodiimide to promote the coupling of glycine ethyl ester to the solid-phase carboxylates. The bound glycine was subsequently released by acid hydrolysis and was quantified by using an amino acid analyzer. Miron and Wilchek (*5*) showed that NHS derivatives of solid-supported carboxyl groups can be quantified by treating the support with hydroxylamine and spectrophotometrically measured the displaced NHS. Under alkaline conditions, the ionized NHS absorbed strongly at 260 nm. However, the preparation of NHS derivatives must be conducted in organic solvents under anhydrous conditions.

In this communication, I describe a simple colorimetric procedure for quantifying the coupling capacity of solid-supported carboxyl groups, i.e., the reactive carboxyl groups. The procedure used four readily available chemicals: (a) cystamine; (b) 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC); (c) dithiothreitol (DTT) or other reducing agents; and (d) 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB).

PRINCIPLE

Figure 1 summarizes the reactions involved in the procedure. Carboxyl groups carrying solid supports that were washed with distilled water were suspended in an aqueous solution of cystamine, pH 4–4.5. To this solution was added an excess amount of water soluble carbodiimide to promote the coupling of the carboxylates with the amino groups of cystamine. An hour later another portion of the carbodiimide was again added. The pH was maintained at 4–4.5 by adding dilute HCl. After 20 h at room temperature, the solid support was washed to remove uncoupled cystamine. The washed solid support, which contained disulfide bonds of the covalently attached cystamine, was treated briefly with a reducing agent, such as DTT, to cleave the disulfide bonds and generate sulfhydryl groups. For every one carboxyl group reacted with the amino group of cystamine, a single sulfhydryl group is introduced. The sulfhydryl groups of the solid support is quantified colorimetrically after reacting with Ellman's reagent, DTNB.

MATERIALS AND METHODS

Chemicals

Cystamine dihydrochloride and ethylenediaminetetraacetic acid (EDTA) were from Aldrich Chemical Co. (Milwaukee, WI). 1-Ethyl-3(3-dimethylaminopropyl)-carbodiimide-HCl and DTNB were from Sigma Chemical Co. (St. Louis, MO). Sepharose CL-4B and CM-Sepharose

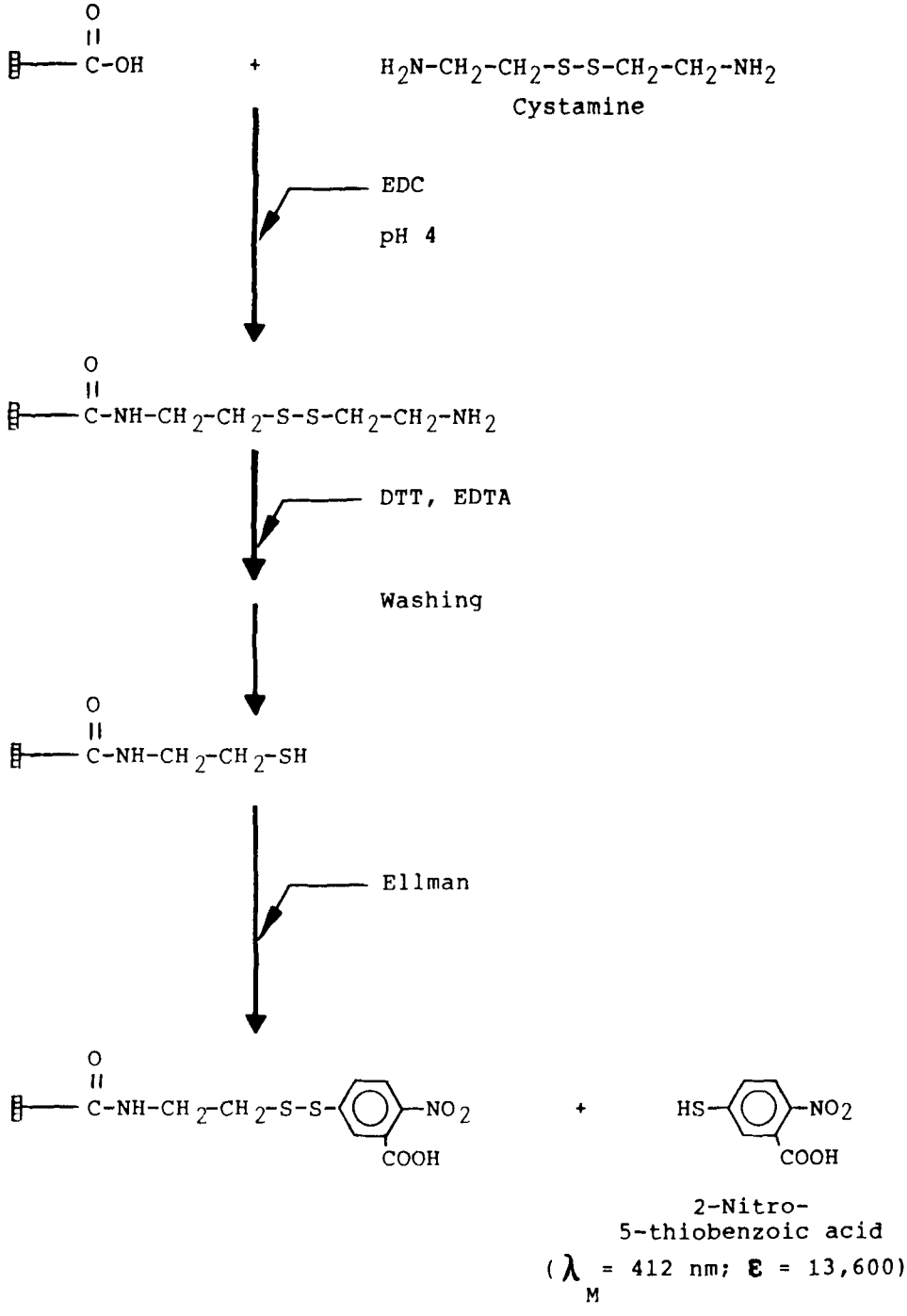

Fig. 1. Principle and reactions in the colorimetric determination of coupling capacity of solid-supported carboxyl groups.

CL-6B were from Pharmacia Fine Chemicals (Piscataway, NJ). Fractogel TSK HW 75(F) was from EM Science (Cherry Hill, NJ). Trisacryl GF 2000 was from LKB (Gaithersburg, MD). Carboxy-Terminal Avid-Gel was from BioProbe International Inc. (Tustin, CA).

Reagent Solutions

Cystamine

60 mM of cystamine was prepared in distilled water, and the pH adjusted to 4–4.5.

Phosphate Buffer

The buffer consisted of 0.1*M* phosphate, pH 7.5, containing 0.15*M* NaCl and 1 m*M* EDTA.

DTT

60 mM of DTT was prepared in the phosphate buffer.

DTNB

50 mM of DTNB was prepared in 0.1*M* phosphate buffer containing 0.15*M* NaCl and the pH adjusted to 8 with NaOH.

Procedure

Wet beads or gel was washed five times with 10 vol of distilled water. The washed gel (0.25–0.5 mL) was suspended in 5 mL cystamine solution, pH 4–4.5, in a plastic tube. The test tube was stoppered and tumbled end-over-end at room temperature for 5 min. Then, 500 μmol EDC was added to the gel suspension and gently stirred for 10 min. During this time the pH of the suspension was continuously monitored and maintained at 4–5 by adding HCl. After 10 min the gel was tumbled end-over-end for 50 min before adding another portion of EDC. Again, for the first 10 min after adding EDC, the suspension was maintained at pH 4–5. The gel was then tumbled at room temperature overnight (~ 20 h). After washing the gel five times with 10 vol of the phosphate buffer, 20 vol of DTT were added to the gel, and it was tumbled at room temperature for 30 min. The gel was thoroughly washed with 5 × 20 vol of the phosphate buffer to remove any trace of DTT. To the washed gel was added 25 vol of DTNB, and, after tumbling at room temperature for 30 min, the gel suspension was centrifuged at 2000 rpm for 10 min. An aliquot of the supernatant was removed and diluted with the phosphate buffer (20–50-fold dilution), and the absorbance of the resultant 2-nitro-5-thiobenzoic acid in the supernatant was read at 412 nm using a similarly diluted DTNB as the blank. The molar extinction coefficient of 2-nitro-5-thiobenzoic acid was 13,600 (*6*).

RESULTS AND DISCUSSION

Using the above-described procedure, I measured the coupling capacity or the concentration of the reactive carboxyl groups of five solid supports, two of them (CM-Sepharose CL-6B and Carboxy-Terminal Avid-Gel) are known to contain significant amounts of reactive carboxyl

groups. The results are presented in Table 1. As expected the Sepharose CL-4B did not show any measurable amount of reactive carboxyl groups. Fractogel showed the presence of carboxyl groups at a very low level. Trisacryl GF 2000 also showed the presence of a low, but slightly higher, concentration of carboxyl groups than that of Fractogel. The trace amount of carboxyl groups in Trisacryl GF 2000 may possibly result from the hydrolysis of some amide bonds of the *N*-acryloyl-2-amino-2-hydroxylmethyl-1,3-propane-diol, which is the monomeric unit of the polymer. The hydrolyzed amides generated some carboxyl groups on the polymer, whereas the amino portions were detached from the polymer. Carboxyl-Terminal Avid-Gel and CM-Sepharose CL-6B, which were known to contain carboxyl groups, indeed showed significant coupling capacity with the presence of reactive carboxyl groups at 7.6 and 107.4 μmol/mL gel, respectively. The manufacturer of CM-Sepharose CL-6B indicated the presence of 12 ± 2 mEq of carboxyl groups/100 mL gel, which is equivalent to 120 ± 20 μmol/mL gel. Within experimental errors, these values are essentially identical. The results do provide direct evidence for the validity of the present, simple method.

CONCLUSION

In conclusion, I have described a new, nonisotopic, simple, and convenient colorimetric procedure for measuring the coupling capacity, i.e., concentration of the reactive carboxyl groups of solid supports. All reagents used in the procedure, cystamine, EDC, DTT, and DTNB, are readily available from commercial sources. Unlike previous methods, the present method does not require the use of radioactive isotopes nor does it require expensive instrumentations, such as an amino acid analyzer or a pH titrator. Furthermore, the procedure is performed in aqueous solution without the need of an anhydrous condition.

TABLE 1
Colorimetric Determination of the Coupling Capacity of Solid-Phase Supported Carboxyl Groups

Solid support	Absorbance at 412 nm	Carboxyl group coupling capacity, μmol/mL gel
Sepharose CL-4B	0	0
Fractogel TSK HW 75(F)	0.011	0.16
Trisacryl GF 2000	0.043	0.63
Carboxy-terminal Avid-Gel	0.52	7.6
CM-Sepharose CL-6B	0.97	107.4

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