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SOLID-PHASE REAGENTS FOR LIQUID CHROMATOGRAPHY DETECTION

JOEL F. STUDEBAKER*
IBM-Thomas J. Watson Research Center, P.O. Box 218, Yorktown Heights, N.Y. 10598 (U.S.A.)

SUMMARY

Methods based on solid-phase reagents have been developed for detecting thiols, disulfides and proteolytic enzymes in liquid chromatography eluates. In each method, compounds in the class of interest release a chromophoric reagent from the packing material in a short column just beyond the analytical column, and that reagent is then detected in an optical detector. It is thus possible to eliminate mixing of the eluate with soluble reagents in mixing tees, the approach used in earlier methods for detecting these classes of compounds. The advantages and limitations of the solid phase approach in the present work are likely to attend attempts to apply the method to other detection problems in liquid chromatography.

INTRODUCTION

In recent years, the applications of reagents bound or adsorbed on solvent-insoluble supports have increased rapidly in number. These solid-phase reagents (SPRs) have been particularly useful in enzymatic and other catalytic processes[1], in synthetic reactions where monofunctional derivatives of polyfunctional compounds or ease of purification are desirable[2,3] and in highly selective affinity separations of biological macromolecules[4]. The successes of affinity separations in low-pressure liquid chromatography (LC) are well known, and high-speed affinity chromatography has recently been introduced by Mosbach's group[5].

In addition to applications in which they are used as the packing material for analytical separations, preparative separations or preliminary cleanup of samples[6], SPRs are potentially useful in other ways in liquid chromatography. We have been particularly interested in using them in detection methods, where they can be used in place of soluble reagents added to column eluates in mixing tees. Our earlier work on using a SPR in a detector for compounds containing disulfides illustrates some of the advantages and limitations which may attend any use of SPRs in LC detection[7]. Here, further development of this detection method is reported.

Because there is no wavelength at which thiols and disulfides may be distinguished from other compounds with an optical detector, samples are usually mixed with a solution of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent[8])

* Present address: IBM/IRD Biomedical Systems, P. O. Box 218, Dayton, N. J. 08810, U.S.A.

which produces the strongly colored DTNB anion on reaction with thiols. Disulfides must reduced to thiols prior to the detection reaction. The reduction step creates a problem when this method is adapted to liquid chromatography of disulfides[9], because it is necessary to add another reagent to complex the reducing agent and thus keep it from producing the DTNB anion itself. In the earlier work[7], we replaced the soluble reducing agent with a column of polymer-bound thiol and thereby eliminated the need to add a complexing agent to remove excess reducing agent after the reduction step (Fig. 1).

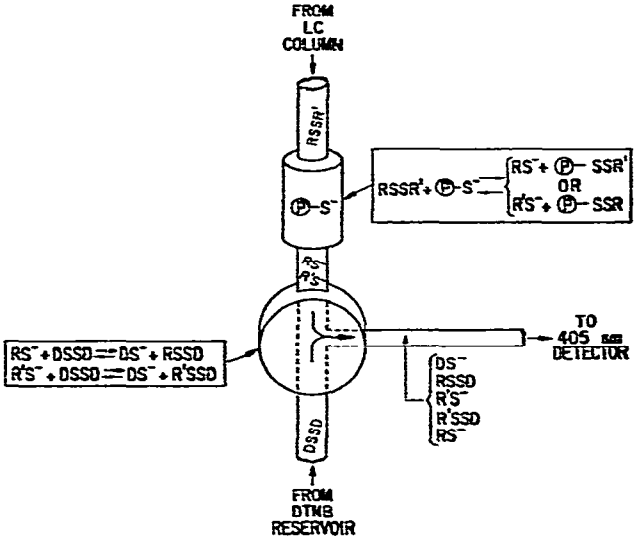
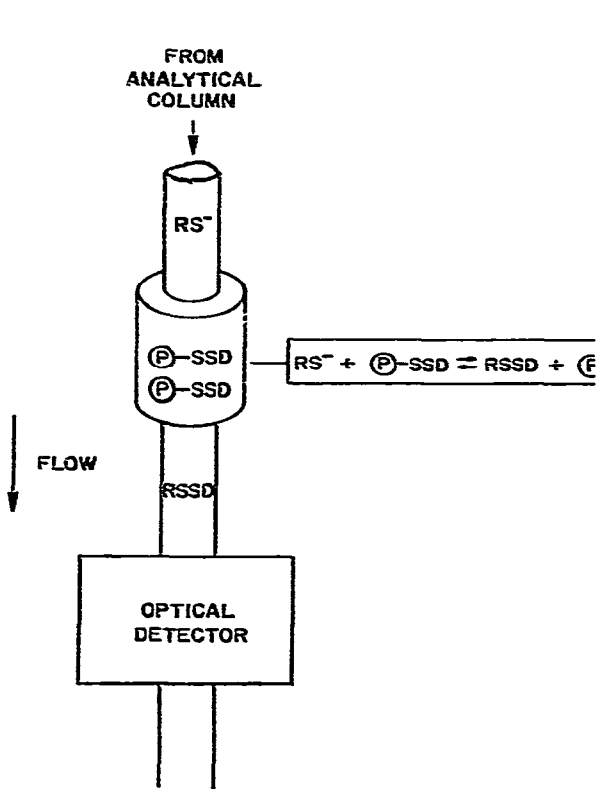


Fig. 1. Diagram of the first apparatus in which a SPR was used for disulfide detection. RSSR' represents a disulfide in the eluate, P–S is the polymer-bound thiol and DSSD is the detection reagent, DTNB. The chemical reactions which take place are indicated in the boxes. (From ref. 7, by permission.)

It seemed attractive to develop this approach further by replacing the DTNB solution with an SPR containing a chromophoric reagent which would be released into the stream entering the detector only in the presence of a thiol. A packing material in which covalently bound thiol groups form mixed disulfides with a strong chromophore like N-dinitrophenylcysteine (DNP-cysteine) is suitable. A thiol in the eluate from the analytical column undergoes a disulfide interchange reaction with the polymer-bound disulfide, so that either DNP-cysteine or its mixed disulfide with the thiol in the eluate exits from the SPR column (Fig. 2). When a column of packing material with free thiol side chains is placed in the line before the column with the bound DNP-cysteine, an all-solid phase system for detecting disulfides in LC eluates results (Fig. 3).

Hydrolytic enzymes may also be detected with SPRs. A hydrolyzable substrate, bound to a solvent-insoluble support and carrying a chromophore at or near its free end, should be cleaved in the presence of the appropriate enzyme (Fig. 4). The sensitivity of the method for a particular enzyme would depend upon the number of chromophoric substrate fragments cleaved by an enzyme molecule during its residence in the column and the sensitivity of the optical detector to the chromophore. Here,



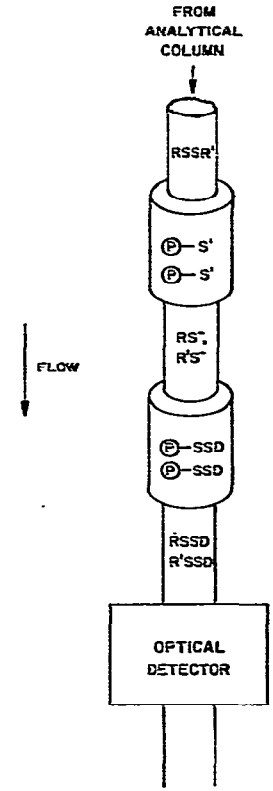


Fig. 2. Diagram of the apparatus in which thiols are detected by the release of a detection reagent which is initially bound to the polymer through a disulfide bond. DS^- represents one half of di-DNP-cystine, and the symbols for the other compounds follow the scheme of Fig. 1.

Fig. 3. Diagram of the all-solid phase apparatus for detection of disulfides. Symbols as in Figs. 1 and 2.

Azocoll[10], a powdered cowhide with an azo dye covalently attached, has been used as a solid phase substrate for the detection of α-chymotrypsin.

EXPERIMENTAL

All experiments were carried out on a Perkin-Elmer LC-55 liquid chromatograph, with the detector wavelength set at 412 nm for detection of thiols and disulfides and 520 nm[10] for the detection of α-chymotrypsin. A 60 cm length of 0.02 in. I.D. stainless-steel tubing was used in place of a column in all cases. The eluent was 0.1 *N* Tris–acetate, 0.001 *M* EDTA, pH 8.0, and it was filtered through a 0.45 μm Millipore filter prior to use.

Cystamine·2HCl (97%) was obtained from Aldrich (Milwaukee, Wisc., U.S.A.), cysteine and di-DNP-cystine from Sigma (St. Louis, Mo., U.S.A.), and α-chymotrypsin (2× recrystallized, 49 units per mg activity) from Worthington Biochemical (Freehold, N.J., U.S.A.). Di-DNP-cystine was first dissolved in methanol (6.8 mg per 0.7 ml methanol), and that solution was then added to 100 ml of aqueous

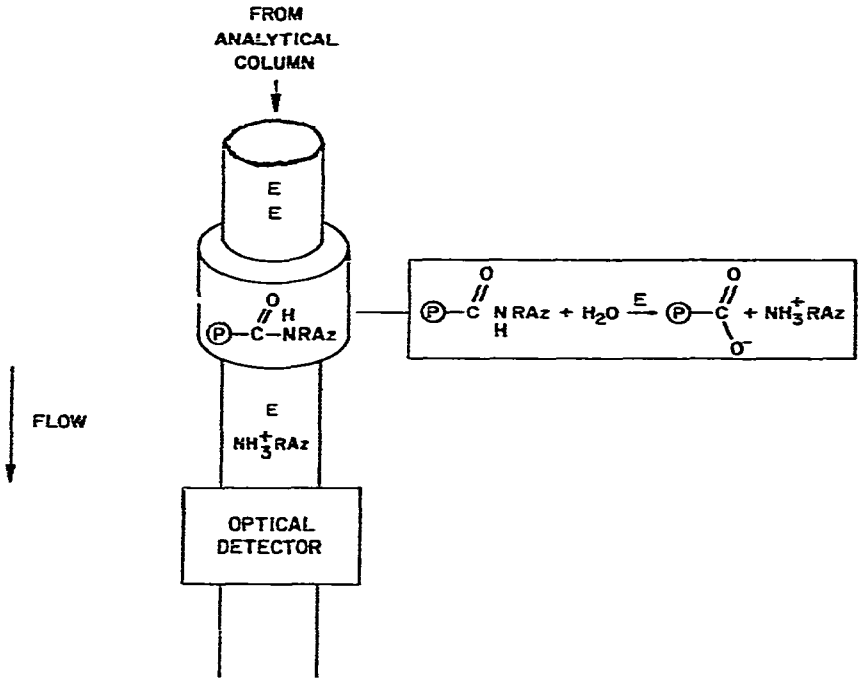


Fig. 4. The system for detecting proteolytic enzymes with a SPR is illustrated here. NH_3^+RAz represents the material released when the amide bond shown is cleaved by the enzyme.

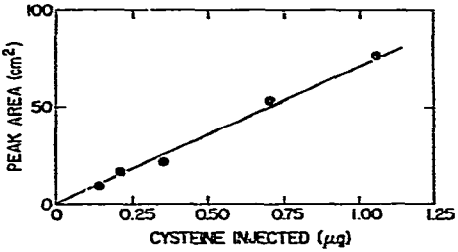
buffer. It was used within 1 week of preparation, and solutions of the other compounds in the list above were prepared the day they were used.

Thiol-Sepharose (Bio-Rad Labs., Richmond, Calif., U.S.A.; 6 μequiv. thiol per ml of hydrated gel) was packed in 4 mm I.D. stainless-steel columns and then washed with a solution of 0.5% β-mercaptoethanol in buffer. The thiol column, 5 cm in length, was obtained by simply washing the β-mercaptoethanol-treated column with buffer until it no longer gave a detectable color when added to an aqueous solution of DTNB. The column containing the mixed disulfide of the polymer-bound thiol and DNP-cysteine was obtained by taking a thiol column, 4 cm in length, and passing 25 ml of the di-DNP-cystine solution through it. DNP-cysteine and di-DNP-cystine which had no reacted were then washed off with buffer. Azocoll (Calbiochem, Los Angeles, Calif., U.S.A.; 50–100 mesh) was swelled in buffer for 2 days at 4°, and then the equivalent of *ca.* 20 mg dry weight was packed into a 4 cm × 4 mm column.

RESULTS AND DISCUSSION

The thiol detector of Fig. 2 gives the curve shown in Fig. 5 when cysteine is used as the test thiol. This response is comparable, on a molar basis, to that obtained with disulfides in the earlier disulfide detector[7]. Buffer alone gives no detectable response. If a fluorescent reagent, like di-Dns-cystine, is used instead of di-DNP-cystine in charging the SPR detection column, a fluorescence detector may be used in place of the optical detector. In light of the inherently greater sensitivity of fluo-

rescence detection methods, the lower limit of detection may be lowered considerably as a result. It is worth noting that neither di-Dns-cystine nor di-DNP-cystine could be used in solutions added in mixing tees to detect thiols. They do not undergo any dramatic change in optical or fluorescence properties which would indicate that the reaction of interest has taken place place, unlike, for example, the esters of dinitrophenol used to detect hydrolytic enzymes. With the SPR method, though, it is only necessary that the reaction result in the breaking of a chemical bond so that the detection reagent is released into the eluate stream when one of the compounds of interest is present.



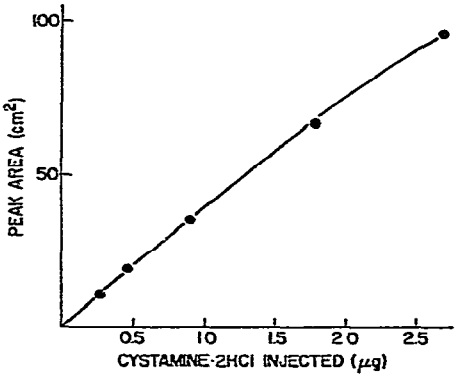
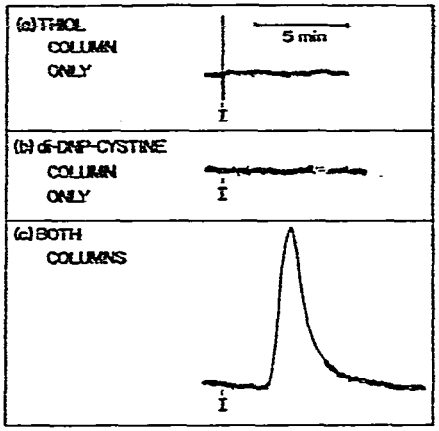


Fig. 5. Response of the apparatus shown in Fig. 2 to samples of the thiol cysteine. The flow-rate was 0.5 ml/min.

Fig. 6. Response of the apparatus shown in Fig. 3 to samples of the disulfide cystamine·2HCl. The flow-rate was 0.5 ml/min.

Fig. 6 shows the response of the all-solid phase disulfide detector to the disulfide cystamine. Again, the sensitivity of the system is comparable to that obtained with the disulfide detector developed earlier. The elimination of excess DTNB in the column eluate, a feature of the present form of the detector, is particularly advantageous when it is desirable to identify the two halves of mixed disulfides. Such identification is necessary in studies of disulfide bond pairing patterns in proteins and protein fragments[11]. In fact, if the disulfide bond between DNP-cysteine and the half-disulfides can be maintained intact, the two halves may be injected into another analytical LC column for identification and be detected by the optical absorption of the DNP group. Thus it might not be necessary to make new derivatives for identification. Fig. 7 shows a control experiment which indicates that the reaction expected is taking place. With only one of the two columns in the line in front of the optical detector, cystamine gives no detectable response. With both in the line, the response shown in (c) is obtained. The sites on the SPRs are gradually used up as the disulfides pass through, but both may be regenerated by the chemical procedures used to prepare them initially.

Detection of α-chymotrypsin with the Azocoll detector is illustrated in Fig. 8. The peaks tail badly when this detector is used, so peak heights rather than peak areas are used in the curve. The most likely explanation for the tailing is that the enzyme is being retarded by affinity chromatographic effects, because there are many



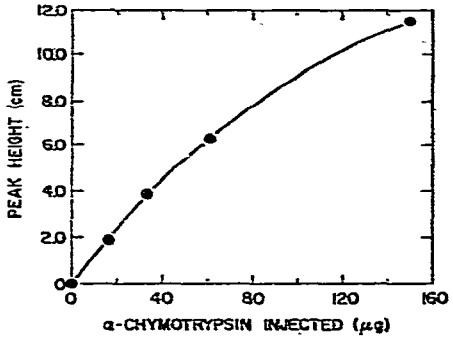


Fig. 7. (a) Record of the response of the optical detector, set at 412 nm, to the disulfide cystamine × 2HCl when the thiol column (P–S⁻ in Fig. 3) alone is in the line before the detector; (b) record of the detector response to 0.9 μg cystamine·2HCl when only the di-DNP-cystine column (P–SSD) is in the line before the detector and (c) response of the both the thiol and di-DNP-cystine columns are in the line.

Fig. 8. Response of the system illustrated in Fig. 4 to samples of α-chymotrypsin. The flow-rate was 0.8 ml/min.

chains in the Azocoll which bind the enzyme but are resistant to hydrolysis by it. This phenomenon illustrates a general problem which can occur in other applications of SPRs in LC detection. Though short in comparison to the analytical columns, the SPR detection columns may affect the observed chromatographic behavior of the compounds separated. Substrate SPRs, once exhausted, must be prepared anew by coupling fresh samples of packing and substrate. Because it is not necessary to add the substrate to all the fractions in a run, though, lower substrate consumption is obtained. Specially synthesized, highly specific substrates or radioactive ones might then be used.

Enzymes can also be used as catalysts for reactions in the detection process, and both soluble[12] and immobilized[13] enzymes have already been used in that way. In addition to substrates, inhibitors of enzymatic reactions may be detected, and cholinesterase has been adapted to the detection of pesticides which inhibit it[14]. Because enzymes are expensive and as catalysts, reusable, it is attractive to use them in the form of SPRs. They are generally inactivated by large concentrations of organic solvents, so, like other SPRs, they place limitations on the eluents which may be used with them. Yet it should be noted, in this regard, that enzymes within a hydrophilic matrix may retain considerable activity when the matrix particles are suspended in an organic solvent[15].

In summary, the present work demonstrates that SPRs can be used as reservoirs for detection reagents as well as a means of conditioning the sample prior to the detection reaction. Several advantages, particularly mechanical simplicity, are gained through the use of SPRs, though limitations are also encountered. These reagents are likely to provide the basis for sensitive and specific methods for the detection of several classes of compounds in liquid chromatography eluates.

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