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Solid-Phase Thiolsulfinates for the Reversible Immobilization of Thiols

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ABSTRACT

A new method for the reversible immobilization of thiol-containing substances on agarose beads is presented. It is based on the use of thiolsulfinate (disulfide monoxide) as a solid-phase reactive group. The thiolsulfinate groups are introduced by controlled oxidation of thiol agarose. The method comprises two steps: First, mild oxidation of the agarose thiol groups to disulfide structures with potassium ferricyanide. Second, the oxidation of the so-formed agarose disulfide groups to thiolsulfinate groups by use of a stoichiometric amount of the oxidizing agent magnesium monoperoxyphtalate.

The solid-phase thiolsulfinate groups react very easily with thiols, which, as a result of the reaction, will be bound to the agarose beads by disulfide bonds. The adsorbent derivative is very suitable for the reversible immobilization of low as well as high-mol-wt thiols as demonstrated with reduced glutathione, penicillamine, mercaptoethanesulfonic acid, thiolated bovine serum albumin, β-galactosidase, and α_1-antitrypsine. Since treatment of the agarose derivatives with an excess of low-mol-wt thiols (e.g., dithiothreitol) leads to release of the bound molecules and regeneration of the original thiol groups, the reactive thiolsulfinate groups can easily be regenerated by the mentioned two-step procedure. The cycle of oxidation, binding, reduction,

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and reoxidation can be performed several times while retaining thiol binding capacity.

Index Entries: Thiolsulfinates; disulfide oxides; gel-bound thiolsulfinates; gel-bound disulfide oxides; agarose thiolsulfinates; magnesium monoperoxyphtalate; thiol immobilization; reversible immobilization; enzyme immobilization.

INTRODUCTION

Solid phases with reactive disulfides of 2-pyridyl disulfide type have, in the past, been the basis for reversible immobilization and covalent chromatography of thiol proteins (*1–3*). In principle, solid-phase-bound disulfide oxides of thiolsulfinate (monoxide) or thiolsulfonate (dioxide) type should show a similar reactivity as reactive disulfides in their reactions with thiols.

We recently found that disulfide oxides can be introduced into thiol agarose by oxidation with H_2O_2 at acidic pH (*4*). The agarose derivatives thus formed showed high reactivity and selectivity toward thiols and could be used for reversible immobilization of both thiol peptides and thiol proteins. Later, we were able to demonstrate that the reactive structures are mainly thiolsulfonate groups. Based on the analytical results, we proposed a mechanism for the activation of gel-bound thiols with aliphatic disulfide and thiolsulfinate structures as intermediates, and a mechanism for coupling involving disulfide bond formation and the concomitant formation of one gel-bound sulfinate group per bound thiol molecule (Fig. 1A,B). We also found that the thiolsulfonate gel can be regenerated to a certain extent by reduction with, e.g., dithiothreitol (DTT) at alkaline pH (Fig. 1C) followed by the aforementioned oxidation procedure (*5*).

However, since 50% of the original thiol groups are converted into sulfinate groups (which can not easily be reduced to thiols) as a result of the immobilization of a thiol-compound on the gel, the number of reactive thiolsulfonate groups and thus capacity to bind thiols decreases by about 50% with each regeneration cycle (*5*). In practice, therefore, it is only meaningful to reactivate the solid phase a few times. In contrast to the thiolsulfonates, aliphatic thiolsulfinates form a sulfenic acid group in their reaction with a thiol. This group can easily be converted back to a thiol (*6,7*) by reaction with an excess of a mild reducing agent [Eq. 1, a–c].

$$\underset{\text{Thiolsulfinate}}{R - S(=O) - S - R} + R'SH \rightarrow \underset{\text{Sulfenic acid}}{R - SOH} + R - S - S - R' \quad (1a)$$

$$R - SOH + R'SH \rightarrow R - S - S - R' + H_2O \quad (1b)$$

$$R - S - S - R' + R'SH \rightarrow RSH + R' - S - S - R' \quad (1c)$$

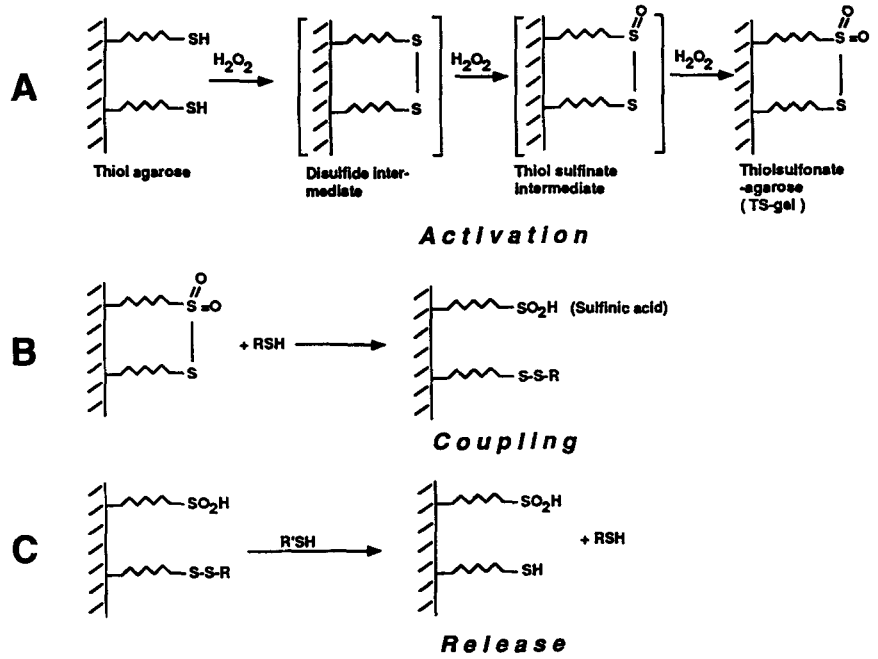

Fig. 1. (A) Formation of thiol-reactive adsorbents by hydrogen peroxide oxidation of thiol agarose. (B) Coupling of thiol compounds to thiolsulfonate agarose. (C) Release of immobilized thiols by excess of reducing agents.

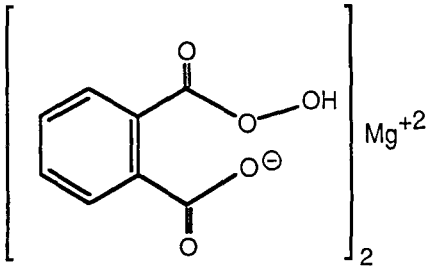

Fig. 2. Magnesium monoperoxyphtalate.

This knowledge led us to search for a way to specifically introduce thiolsulfinate moieties into agarose and other solid phases. The oxidizing agent magnesium monoperoxyphtalate was found to be a suitable reagent (Fig. 2). With this reagent, it is possible to introduce only one oxygen atom per immobilized aliphatic disulfide group, thus forming a thiolsulfinate moiety. Agarose thiolsulfinate derivatives (TSI-gel) prepared with monoperoxyphtalate showed the same characteristic selectivity and reactivity toward thiols as the thiolsulfonate derivatives (TS-gel) prepared by H_2O_2 oxidation. Because the regeneration of the reactive groups is close to 100%, the same gel batch can be reused a large number of times. These properties make the thiolsulfinate an interesting alternative to the thiolsulfonate

group as a solid-phase reactive moiety, especially in applications where the possibility of reusing the solid phase material is of importance.

EXPERIMENTAL

Materials

Sepharose 4B, PD-10 columns (Sephadex G-25) and *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) were supplied by Pharmacia BTG-LKB, (Uppsala, Sweden). Epichlorohydrine (1-chloro-2, 3-epoxy propane), 2,2′-dipyridyl disulfide, reduced glutathione (GSH), dithiothreitol (DTT), valine, penicillamine, mercaptoethanesulfonic acid sodium salt (MESNA), *o*-nitro-phenyl β-D-galactopyranoside (ONPG), bovine serum albumin (BSA), β-galactosidase from *E. coli* and α_1-antitrypsine were purchased from Sigma (St Louis, MO). Perhydrol (30% hydrogen peroxide), potassium ferricyanide, and magnesium monoperoxyphtalate were from Merck (Darmstadt, Germany). All other chemicals used were of reagent or analytical grade.

Preparative Methods

Preparation of Thiol-Agarose

The preparation of mercaptohydroxypropyl ether agarose (thiolagarose) was carried out essentially as described by Axén et al. (*8*). An amount of 15 mL epichlorohydrine/100 g suction dried Sepharose 4B and 100 mL 1*M* NaOH was normally used.

Thiol Group Analysis

The thiol content of both soluble and insoluble material was spectrophotometrically determined by titration with 2,2′-dipyridyl disulfide dissolved in 0.1*M* sodium phosphate buffer pH 8.0, according to Brocklehurst et al. (*1*).

Preparation of Thiolsulfinate-Agarose (TSI-Gel)

DISULFIDE-AGAROSE (S_2-GEL)

Fifty grams of suction-dried thiol-agarose was suspended in 100 mL 0.1*M* sodium phosphate buffer pH 7.0 and 0.1*M* potassium ferricyanide was added by 1.0-mL aliquots while shaking until the yellow color persisted for at least 30 min. The gel was then thoroughly washed on a sintered glass filter with buffer, 1*M* NaCl, and 0.2*M* sodium acetate buffer pH 5.0.

THIOLSULFINATE-AGAROSE (TSI-GEL)

Fifty grams of suction dried S_2-gel (*see above*) was suspended in 100 mL 0.2*M* sodium acetate pH 5.0 in which the required amount of magnesium monoperoxyphtalate had been dissolved (0.5 moles/mole S-S groups). The suspension was incubated while shaking for 2 h at 50°C. The gel

derivative was then thoroughly washed on a sintered glass filter with 50 m*M* sodium acetate buffer pH 5.0 and 0.1*M* acetic acid. The washed gel was stored at 4°C as a suspension in 0.2*M* sodium acetate buffer pH 5.0.

Preparation of and Coupling to Thiolsulfonate-Agarose (TS-Gel)

This was performed as previously described (5).

Titration of Thiol-Reactive Structures in the Agarose Derivatives

One and a half to two and a half grams suction-dried gel aliquots (TSI, TS, or disulfide gels) were equilibrated with 0.1*M* sodium phosphate buffer pH 7.0 in centrifuge tubes. The amount in each tube was adjusted to 3.0 g with mentioned phosphate buffer. Three-milliliter aliquots of 15 m*M* glutathione dissolved in the same buffer were added to each tube while mixing (Vortex). The suspensions were incubated for 30 min at 22°C with mixing every fifth minute. After centrifugation, 50-μL aliquot of supernatants were mixed with 3.0 mL of 0.25 m*M* 2,2′-dipyridyl disulfide dissolved in 0.1*M* sodium phosphate buffer pH 8.0, and the absorbancies at 343 nm were then measured. A blank for spontaneous oxidation of glutathione was run by replacing the gel with an equal volume of phosphate buffer. The dry weights of thoroughly-washed gel derivatives were then determined after drying over phosphorous pentoxide in a dessicator until constant weight. The amounts of glutathione bound to the oxidized gels were calculated from the difference in absorbance readings at 343 nm between the glutathione blank and the gel supernatants. More accurate determinations of the bound glutathione were performed by amino acid analysis after thorough washing and drying of the gel derivatives.

Release of Gel-Bound Peptides and Proteins by Reductive Cleavage

This was performed by incubating the gel derivatives with 100 m*M* DTT in 0.1*M* sodium phosphate buffer pH 8.0 while shaking for 90 min (the amount of reducing solution added was four times by volume the amount of suction-dried gel derivative). The gel derivatives were then thoroughly washed with buffer, 1*M* NaCl, 6*M* urea, and water. The remaining amount of peptide or protein bound to the gel was then determined by total amino acid analysis.

Preparation of Nonmercaptalbumin

Nonmercaptalbumin was obtained as the breakthrough fraction from covalent chromatography of bovine serum albumin (BSA) on activated thiol Sepharose 4B (3).

Thiolation of Bovine Serum Albumin

This was performed as reported elsewhere (5). The obtained thiol content was 4.0 moles of SH groups/mole BSA.

Repetitive Use of TSI-Gel (After Regeneration) for Reversible Immobilization of Thiol Proteins

IMMOBILIZATION OF THIOLATED BSA

Suction-dried TSI-gel aliquots (either without previous use or after the first or second regeneration) were incubated in plastic tubes with screw caps with thiolated bovine serum albumin solution (10 mg/mL) in 0.1*M* sodium phosphate pH 7.0. The amount of bovine serum albumin used in each case was equivalent to 50%, the starting capacity of the gel. After slowly rotating the tubes end-over-end at RT for 24 h, the insoluble derivatives were thoroughly washed with coupling buffer, 1*M* NaCl, 6*M* urea, and distilled water. Small samples of the gel derivatives were dried in a dessicator over P_2O_5 and subjected to amino acid analysis.

REGENERATION OF USED TSI-GEL

After release of bound material from the gel derivatives by reductive cleavage and thorough washing, reactivation was performed according to the two-step procedure described above. Thiol-reactive moieties recovered after each cycle were determined by titration with glutathione (*see above*).

Immobilization of Low Molecular Weight Thiols

One and a half grams suction dried TSI-gel aliquots were equilibrated with 0.1*M* sodium phosphate pH 7.0. The volume of the suspensions were adjusted to 3.0 mL with buffer and 3.0 mL 15 m*M* solutions of glutathione, mercaptoethanesulfonic acid sodium salt, or penicillamine were added. The suspensions were incubated for 30 min at RT with mixing every fifth minute (Vortex). The amount of gel-bound thiols was estimated by back titration with 2,2′-dipyridyl disulfide as explained above (*see* titration of thiol reactive structures in agarose derivatives). More accurate determinations were performed by amino acid analysis (glutathione and S/N elemental analysis [mercaptoethanesulfonic acid and penicillamine]) of the thoroughly washed derivatives, dried over P_2O_5 in a dessicator. With the specific purpose of testing the selectivity of the TSI-gel toward thiols, incubation was also performed under similar conditions with valine, which is structurally similar to penicillamine but lacks the thiol group. Gel-bound valine was determined by nitrogen analysis.

Immobilization of Thiolproteins

1. Six and a half milliliter thiolated bovine serum albumin (10 mg/mL) in 0.1*M* sodium phosphate pH 7.0 was incubated with 1.5 g suction-dried TSI-gel (containing 390 μmoles reactive groups/g dried gel) during 24 h at RT in plastic tubes with screw caps, while rotating end-over-end. With the specific purpose of testing the lack of reactivity of TSI-gel toward nonthiolproteins, incubation under similar conditions was also performed with nonmercaptalbumin. After incubation, the

insoluble derivatives were thoroughly washed with coupling buffer, 1*M* NaCl, 6*M* urea, and distilled water. The gel derivatives were dried in a dessicator over P_2O_5 and subjected to amino acid analysis.

2. α_1-Antitrypsine (10 mg/mL) in 0.1*M* sodium phosphate pH 7.0, was incubated with 5 m*M* dithiothreitol for 1 h at RT. After reduction, dithiothreitol was removed by desalting on PD-10 columns. Four milliliter reduced α_1-antitrypsine (2 mg/mL) were then incubated with 1.5 g suction-dried TSI-gel as described above. After the incubation the insoluble derivatives were processed in the same way as in 1.
3. Six and a half milliliter β-galactosidase solution (3.0 mg/mL) in 0.1*M* potassium phosphate pH 7.0 was mixed with 1.5 g suction dried TSI-gel (containing 390 μmoles reactive groups/g dried gel) and slowly rotated end-over-end at 4°C for 16 h. After thorough washing of the immobilized enzyme derivative with the coupling buffer, the coupling buffer with 0.5*M* NaCl and distilled water, one aliquot was dried in a dessicator over P_2O_5 for amino acid analysis while another aliquot was suspended in activity buffer for assay of gel-bound β-galactosidase activity.

Determination of Immobilized β-Galactosidase Activity

This was performed at pH 7.5 (0.1*M* potassium phosphate) with *o*-nitrophenyl β-D-galactopyranoside (ONPG) as the substrate (*9*).

RESULTS AND DISCUSSION

Preparation of Thiolsulfinate Gels

Treatment of thiol agarose with the oxidizing agent hydrogen peroxide leads to formation of solid-phase bound disulfide oxide groups (*5*). Although this reaction of solid-phase thiols goes through several stages (Fig. 1A). and a small percentage of disulfide and thiolsulfinate groups seem to be present in the final oxidation product, all efforts to stop the oxidation at the thiolsulfinate stage have failed. In order to achieve this, we looked for other oxidation agents, and magnesium monoperoxyphtalate seemed an interesting candidate. This rather recently introduced oxidizing agent has been successfully used to convert thioethers to sulfoxides and sulfones (*10*). It can donate two oxygen atoms/mole under mild conditions (i.e., each mole would be able to oxidize four equivalents of reducing agent).

We first attempted to oxidize thiol agarose directly with magnesium monoperoxyphtalate by using 0.5 moles of oxidizing agent/mole of gel-bound thiol. The obtained agarose derivative, however, showed almost no reactivity towards thiols and titration showed the presence of a considerable amount of strongly acidic groups. This indicates that oxidation of the solid-phase thiols with magnesium monoperoxyphtalate gives sulfinic or sulfonic acid residues directly without proceeding via the disulfide and disulfide oxide intermediates.

In a second series of experiments, we tried an alternative approach in which the thiols first were converted into gel-bound disulfide groups (S_2-gel) by gentle oxidation with potassium ferricyanide at neutral pH (Fig. 3A). This reagent is known to be a mild oxidizing agent and has, e.g., been used to oxidize dithiothreitol (*11*) and cytochrome C (*12*) to their disulfide forms. Since no free thiol or acidic groups could be detected after the incubation with the ferricyanide, we assumed that the S_2-gel had a content of disulfide structures equal to 50% of the original thiol group titer. We then attempted to oxidize the agarose-disulfide groups further to the disulfide monoxide (thiolsulfinate) stage by using the corresponding stoichiometric amount of magnesium monoperoxyphtalate (i.e., 0.5 moles/mole of disulfide groups) (Fig. 3B). Thus, starting with a thiol-agarose containing 712 μmoles of thiol groups/g dried gel and following the procedure outlined above, we obtained a thiol reactive derivative containing 388 μmoles of reactive groups/g dried gel as estimated by titration with reduced glutathione (Table 1). The disulfide-agarose derivative, on the other hand, scarcely reacted with glutathione at all, thus demonstrating that further oxidation of the disulfide groups was necessary.

The almost complete absence of free thiols after the first oxidation (with potassium ferricyanide) is a bit surprising. It seems reasonable that at least some of the thiol groups, owing to their location in remote areas of the gel network, would be hindered from coming close enough to other thiols to form disulfide groups.

One explanation might be that the thiols introduced on agarose caused by the thiolation procedure and the special microcrystalline structure of this gel (the polymerchains are wound up in a ropelike structure with large vaccuoles in between) end up in sterically favorable positions for reactions with each other. The zero thiol titer might, of course, also be caused by the bulky 2,2'-dipyridyl disulfide (the thiol titrating reagent) failing to react with less exposed thiol groups.

Properties of Thiolsulfinate Gels

Some characteristics of the behavior of the thiol reactive gel prepared with the new, two-step method were compared with those of the thiol-sulfonate-gel (TS-gel) prepared by H_2O_2 oxidation, with both gels having been obtained from the same thiol-agarose batch (Table 1). The amount of bound glutathione remaining on the gels after treatment with dithiothreitol

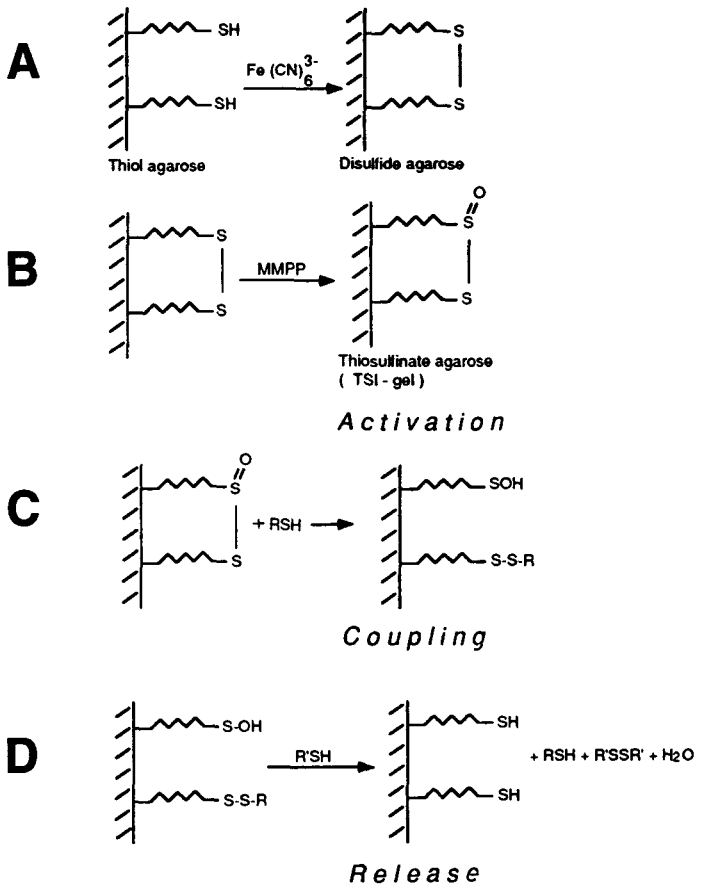

Fig. 3. Synthesis, properties and regeneration of solid phase thiolsulfinates. (A) Oxidation of thiol agarose to disulfide gel, with potassium ferricyanide at pH 7.0. (B) Oxidation of disulfide to thiolsulfinate moieties by a stoichiometric amount of magnesium monoperoxyphtalate (0.5 moles/mole of disulfide moieties) in 0.2*M* sodium acetate pH 5.0 at 50°C. (C) Proposed mechanism for the coupling of thiols to TSI-gel at neutral or weakly acidic pH. (D) Release of gel-bound thiols by reduction of disulfide bonds with an excess of low molecular weight thiols (e.g., 100 m*M* dithiothreitol in 0.1*M* sodium phosphate pH 8.0), thus giving total regeneration of solid phase thiol groups. (E) After step (d), regeneration of TSI-gel can be performed following the two-step procedure outlined in (a) and (b).

was zero for both derivatives. This result confirms the disulfide nature of the bonds formed between the glutathione and both types of activated solid phase.

When treating the glutathione agarose derivative prepared from thiolsulfonate-agarose with an excess of dithiothreitol, the thiol content of the gel derivative obtained was only 52% of the value of the original thiol agarose gel (Table 1). In contrast the corresponding figure for the reduced glutathione-TSI-gel derivative was 96%. This strongly indicates that the

Table 1
Comparative Properties of Thiolsulfonate Agarose (TS-Gel) and Thiolsulfinate Agarose (TSI-Gel)

Gel type	Gel bound SH groups: μmoles/g Dried gel	Gel bound SH groups: Percent of starting value	Amount of GSH bound, μmoles/g dried gel	Amount of GSH after DTT treatment, μmoles/g dried gel
SH-gel	712	100	0	—
S_2-gel	< 10	—	31	—
TSI-gel	< 10	—	388	0
TSI-GSH, DTT reduced[a]	683	96	0	—
TS-gel	< 10	—	382	0
TS-GSH DTT reduced[a]	370	52	0	—

[a] Glutathione derivatives after 1 h incubation with 100 m*M* DTT in 0.1*M* sodium phosphate pH 8.0, followed by thorough washing with the same buffer.

Abbreviations: GSH = Glutathione; TSI = Thiolsulfinate; DTT = Dithiothreitol; TS = Thiolsulfonate; S_2 = Disulfide; SH = Thiol.

Table 2
Repetitive Use of Thiolsulfinate Agarose (TSI-Gel) (by Regeneration) for Reversible Immobilization of Thiolproteins (Thiolated BSA)

Step	Gel bound SH groups, μmoles/g dried gel	Thiol-reactive groups, μmoles/g dried gel	Bound BSA,[a] mg/g dried gel
SH-gel	1027	—	—
TSI-gel first use	0	415[b] (362)[c]	133
TSI-BSA derivative after DTT	1083	0	5.5
TSI-gel after second regeneration (third use)	0	545[b] (383)[c]	132
TSI-BSA derivative after DTT	—	0	19

[a] Amounts of thiolated BSA corresponding to approx 50% of the gel's binding capacity were used.

[b] By thiol titration.

[c] By amino-acid analysis.

Abbreviations: BSA = Bovine serum albumin; DTT = Dithiothreitol; SH = Thiol.

Table 3
Comparative Immobilization of Thiols
and Nonthiol Compounds on Thiolsulfinate-Agarose (TSI-Gel)

Gel derivative	S%	N%	Amount bound, μmol/g or mg/g dried derivative
Untreated TSI-gel control	3.3	0.06	—
Nonthiol Compounds			
Valine	3.3	0.06	0
Nonmercaptalbumin	—	—	0
Low molecular weight thiols (μmoles/g)			
GSH	—	—	415 (388)[a]
MESNA	6.2	—	438
Penicillamine	4.8	0.77	468
Thiol proteins (mg/g)			
α_1-antitrypsine[b]	—	—	53.4
β-galactosidase[b]	—	—	122.8
BSA (thiolated)	—	—	276

[a]By amino acid analysis.
[b]No saturation conditions.
Abbreviations: GSH = Glutathione; MESNA = Mercaptoethanesulfonic acid; BSA = Bovine serum albumin.

reactive groups introduced into the thiol agarose as a result of the two-step oxidation really are disulfide monoxide (thiolsulfinate) groups (Fig. 3B). Furthermore the high recovery of thiol groups in the case of TSI-gel derivatives makes possible the full regeneration of the reactive structures. We have demonstrated the feasibility of repetitive use through regeneration of the same batch of thiolsulfinate-agarose (TSI-agarose) using thiolated bovine serum albumin as a model substance. The amount of thiol reactive groups (thiolsulfinate groups) as well as the thiol protein binding efficiency remained almost constant through three cycles of oxidation, coupling, reductive release, and reoxidation (Table 2). Thiolated bovine serum albumin could also, after immobilization, be released from the agarose by reduction with dithiothreitol (Table 2). The small amounts of protein remaining on the gel after reduction (4% after the first and 15% after the third cycle) can perhaps be explained by multipoint, noncovalent interactions between the albumin and the agarose. In addition to glutathione and thiolated bovine serum albumin, the usefulness of the TSI-agarose derivatives was also demonstrated with the low-mol-wt thiols mercaptoethanesulfonic acid and penicillamine, as well as with the high-mol-wt thiols β-galactosidase and reduced α_1-antitrypsine (Table 3). Based on the reproducibility of the obtained results, titration of solid-phase thiol reactive structures could be performed with any of the low molecular weight thiols.

We, however, decided to continue using glutathione since this thiol is easily available and previously has been established as a titration agent (*4,5*). In the case of native *E. coli* β-galactosidase, which contains several nonessential exposed thiol-groups, immobilization of high amounts of protein proceeded with good yields (around 40% of added protein). The β-galactosidase activity of the nonbound protein was rather low, whereas about 70% of the originally applied activity was found in the material released from the gel by treatment with dithiothretol. The immobilization of β-galactosidase on the thiolsulfinate agarose thus proceeded with a concomitant purification. A similar purification effect observed when immobilizing β-galactosidase on thiolsulfonate agarose will be described and discussed more in detail elsewhere.

The selectivity of the thiolsulfinate gel (TSI-gel) for thiols was demonstrated by reacting it under identical conditions with penicillamine and valine (an aminoacid that has an analogous structure to penicillamine but lacks thiol group). Although the former substance was quantitatively bound to the activated agarose, valine did not bind at all (Table 3). The thiol selectivity was also demonstrated with proteins. Thus, complete absence of protein was demonstrated after incubation of TSI-gel with non-mercaptalbumin at pH 7.0 for 20 h followed by extensive washing to remove noncovalently bound protein. On the other hand, when the same experiment was repeated with thiolated bovine serum albumin, considerable amounts of protein bound to the solid phase (Table 3). Native bovine serum albumin containing 48% mercaptalbumin was also incubated with the reactive gel. In this case much less protein (although significant amounts) bound to the solid phase. This most probably is owing to the fact that the native thiol group is located at the bottom of a pocket of the protein and is thus not very accessible for reaction with the thiolsulfinate groups. A similar effect has previously been observed with thiolsulfonate agarose-containing gels (*5*). In that case, the steric inhibition was even more pronounced, possibly because the thiolsulfonate groups contain two oxygen atoms, as compared to thiolsulfinate groups that have only one oxygen atom.

Comparison of Thiolsulfinate (TSI) and 2-Pyridyl Disulfide as Reactive Groups on Agarose

The thiolsulfinate-group shows, as does the 2-pyridyl disulfide group (*1–3*) very high specificity for reaction with thiols. Its reactivity with exposed aliphatic thiols is also very high. In fact, it is possible to convert agarose bound thiolsulfinate-groups to 2-pyridyl disulfide groups at pH 5–7 by reaction with 2-thiopyridone. With less exposed thiols (e.g., the native thiol in mercaptalbumin), however, the gelbound thiolsulfinate-group shows lower reactivity than the 2-pyridyldisulfide. This property

can possibly be used for the separation of proteins with exposed thiol-groups from those with buried thiols. The gelbound TSI-groups have the same stability as a function of pH and time when stored at neutral pHs as the 2-pyridyldisulfide groups. Unlike the latter group, the thiolsulfinate is also stable in the presence of sodium azide, a bacterioside often used to prevent bacterial growth in gels. Such a stability toward nucleophilic azide ion has previously been observed for gelbound thiolsulfonate groups (*5*). The major difference between the TSI and 2-pyridyldisulfide is that when the former structure is used as a solid phase reactive group, there is no release of any low-mol-wt thiol compound from the agarose as a result of immobilization of a thiol, since the formed sulfenate group is bound to the agarose. Reaction with 2-pyridyldisulfide, on the other hand, leads to a release of the low-mol-wt compound 2-thiopyridone. This is a definite advantage in applications where the activated agarose is utilized to remove unwanted thiols from a solution. However, the course of the immobilization can not be followed as conveniently as when 2-pyridyl disulfide agarose is used since in this case the 2-thiopyridone released as a result of the reaction can easily be spectrophotometrically detected and quantified (2-thiopyridone: λ_{max} = 343 nm, E = 8.060 × $10^3 M^{-1} cm^{-1}$). The properties of thiolsulfinate agarose are very similar to the ones recently reported for thiolsulfonate agarose (*4,5*), except that the latter gel can be regenerated only a few times, due to the formation of gelbound, nonreducible sulfinate groups. The thiolsulfinate agarose as the 2-pyridyl disulfide agarose can, at least in theory, be regenerated an unlimited number of times.

The main characteristics of the new immobilization method are:

1. It is absolutely specific for thiols (under the conditions used).
2. The immobilized material can easily be released by reduction.
3. The activated gel structures are completely regenerable.

Thus the thiolsulfinate group combines the advantages of the previously described 2-pyridyl disulfide and thiolsulfonate groups and should therefore be a useful alternative to these groups as a solid-phase reactive group.

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