0273-2289/91/3102—0175$04.20

A New Method for Reversible Immobilization of Thiol Biomolecules Based on Solid-Phase Bound Thiolsulfonate Groups

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Received April 12, 1991; Accepted May 29, 1991

ABSTRACT

A new method for the reversible immobilization of thiol bimolecules, e.g., thiolpeptides and thiolproteins, to beaded agarose and other solid phases is reported. The method consists of an activation and a coupling step. The activation is based on oxidation of disulfides (or thiol groups via disulfides) present in a solid phase by hydrogen peroxide at moderately acidic pH. This oxidation leads to disulfide oxides (thiolsulfinate groups of which the majority are further oxidized to thiolsulfonate). The thiolsulfonate groups react easily with thiol compounds, which become immobilized via disulfide bonds. The pH range for thiol coupling is wide (pH 5–8), but for most thiols the reaction seems to proceed faster at pH>7. The stability of the reactive group to hydrolysis, especially at neutral and weakly acidic pH, is very high. The activated gel, therefore, can be stored as a suspension at pH 5 for extended periods. The method has been used to reversibly immobilize glutathione, β-galactosidase, alcohol dehydrogenase, urease, and papain, all with exposed thiol groups as well as thiolated bovine serum albumin and sweet-potato β-amylase.

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Depending on the thiol content of starting thiol-agarose, thiolsulfonate-agarose derivatives with different binding capacities can be obtained. Thus, up to 5.0 mg (16 μmol) glutathione and 15 mg thiolprotein/mL gel derivative have been immobilized.

The gel bead can be regenerated and reused at least twice. Besides agarose, cellulose, crosslinked dextran, and polyacrylamide were shown to be very suitable as supports for solid-phase thiolsulfonates.

Index Entries: Protein immobilization; agarose-bound thiolsulfonates; disulfide oxides; thiolpeptides; thiolprotein; reversible immobilization; activated agarose derivatives.

INTRODUCTION

Alifatic thiols and disulfides react by thiol–disulfide exchange reactions under formation of equilibrium mixtures containing both the free thiols and the different disulfide combinations (*1*):

$$RSH + R_1S\text{-}SR_1 = RS\text{-}SR_1 + R_1SH$$

$$RS\text{-}SR_1 + RSH = RS\text{-}SR + R_1SH$$

The reactions proceed at reasonable rates only at alkaline pH, at which the reactive species, the thiolate ions, are present at high concentrations. Solid-phase bound alifatic disulfides, therefore, are not useful as reactive groups for the immobilization of thiol proteins and peptides. However, with a so-called reactive disulfide, such as 2-pyridyldisulfide, the exchange with alifatic thiols under formation of mixed solid-phase bound disulfides is thermodynamically favored. Such a thiol–disulfide exchange reaction also occurs relatively quickly at neutral and acidic pH (*1*). Thus, solid phases (especially beaded agarose) with 2-pyridyldisulfide substituents have been widely used for reversible immobilization and covalent chromatography of thiolproteins and thiolpeptides (*2–6*). A disadvantage with these gel derivatives is that their reactions with thiols leads to concomitant release of 2-thiopyridone, which thus contaminates the nonimmobilized material.

Another way of creating a more S-reactive group is to convert an alifatic disulfide to corresponding thiolsulfinate or thiolsulfonate. A large number of such derivatives have been synthesized by oxidation of low-mol-wt disulfides with oxidizing agents, such as hydrogen peroxide, peracetic acid, potassium permanganate, and potassium meta-periodate (*7*). There are also examples of naturally occurring disulfide oxides, such as allicin (diallyl thiolsulfinate) in garlic (*8*). The thiolsulfonates are usually rather stable in nonreducing conditions at neutral and acidic pH, but react easily with thiols under formation of disulfides and sulfinic acid. In fact the compound called methylmethane thiolsulfonate (MMTS) has been

widely used for reversible blocking of thiol groups in thiol-dependent enzymes (*9,10*). Aromatic thiolsulfonates have also been used for titration of thiols and for blocking of thiol groups in proteins (*11*).

Recently, we have found that disulfide oxide groups also can be introduced into thiol-containing agarose beads by their oxidation with H_2O_2 at acidic pH. The agarose derivates thus formed showed high reactivity toward thiols and could be used for immobilization of both low-mol-wt thiols and thiolproteins (*12*). In this paper we present evidence that the reactive structures are mainly thiolsulfonate groups and, based on the analytical results, propose a mechanism for activation and coupling. We also show that the method is specific for thiol groups in the immobilized peptides and proteins and that the immobilized ligand is released under reducing conditions. Contrary to the reactive disulfide gels mentioned above, the binding of thiols to the thiolsulfonate gels does not lead to release of any soluble compound. The potential application of the thiolsulfonate solid-phase adsorbent for the reversible immobilization of thiol enzymes is finally demonstrated with several examples.

EXPERIMENTAL

Materials

Sepharose 4B, Sephadex G-75, PD-10 columns (Sephadex G-25), beaded cellulose, *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) were supplied by Pharmacia BTG (Uppsala, Sweden). Epichlorohydrin (1-chloro-2,3-epoxypropane), reduced glutathione, 2-thiopyridone, 2,2′-dithiopyridine (2,2′-dipyridyldisulfide, 2-PDS), dithiothreitol (DTT), bovine serum albumin (BSA), β-galactosidase from *E. coli*, urease from jack beans, papain from papaya latex, β-amylase from sweet potato and baker's yeast alcohol dehydrogenase were purchased from Sigma Chemical Company (St. Louis, MO). Perhydrol® (30% hydrogen peroxide) and sodium thiosulfate were products of Merck AG (Darmstadt, Germany). Eupergit C (epoxy-activated polyacrylamide) was from Rohm Pharma GmbH (Darmstadt, Germany). All other chemicals used were of reagent or analytical grade.

Preparative Methods

Thiol-agarose. The preparation of mercaptohydroxypropyl ether agarose gel (thiol-agarose) was carried out essentially as described by Axén et al. (*13*). In this method the agarose beads (Sepharose 4B) are first reacted with epichlorohydrin in alkaline media. The oxirane groups thus formed are then converted with sodium thiosulfate to gel-bound thiosul-

fate groups, which finally are reduced with DTT to thiol groups. The thiol content of the agarose derivative is determined by the amount of epichlorohydrin added in the first step. Thus, agarose derivatives containing from a few up to 900 μmol SH groups/g dried gel can be prepared in a reproducible way. As a standard procedure to obtain a gel suitable for protein immobilization, it is recommended to use 2.5 mL epichlorohydrin/15g suction-dried Sepharose 4B and 15 mL of 1*M* NaOH. These conditions will give a thiol-agarose derivative containing between 400 and 600 μmol thiol groups/g dried gel.

Disulfide-gel. Suction-dried thiol-agarose (15 g) was suspended in 45 mL of 0.1*M* sodium phosphate, pH 8.0, containing traces of cupric ions (Cu^{2+}), and air was bubbled through the suspension for 6 h under shaking. This treatment leads to complete disappearance of all thiol groups under formation of gel-bound disulfide groups as determined by titration with 2,2'-dipyridyl disulfide. Oxidation by air proceeds only to the disulfide stage, as supported by the fact that all gel-bound thiol groups were recovered when the oxidized gel was treated with an excess of DTT. The air-oxidized gel was usually stored in 0.2*M* sodium acetate, pH 5.0, at 4°C and used as a control in the immobilization experiments.

Thiolsulfonate-agarose (TS gel). The recommended procedure based on the optimization is described below. Suction-dried thiol-agarose (15 g, containing between 400 and 600 μmols SH groups/g dried gel) was suspended in 45 mL of 0.2*M* sodium acetate, pH 5.0. Hydrogen peroxide (30%) was added by aliquots under continuous shaking, 1.8 mL initially and 2.2 mL each after 30, 90, and 150 min. The incubation was then continued to give a total reaction time of 30 h. The oxidized gel was then transferred to a sintered-glass filter and washed with 0.1*M* acetic acid until free of hydrogen peroxide. The activated gel was stored in 0.2*M* sodium acetate, pH 5.0, at 4°C until used.

The effects of pH, hydrogen peroxide concentration, and incubation time on the activation of thiol-agarose (430 μmol SH groups/g dried gel) was studied. After activation under the selected conditions, the gel derivatives were washed with 0.1*M* acetic acid until free of reagents and equilibrated in storage buffer (0.2*M* sodium acetate, pH 5.0). The number of reactive structures introduced was then determined by the glutathione method (*see below*).

1. The effect of pH on the activation reaction was studied in the range 2–7. Aliquots of suction-dried thiol-agarose (6.0 g each) equilibrated in water were suspended in 15 mL of the following buffers: 0.2*M* acetic acid (pH 2.1), 0.2*M* sodium acetate (pH 3.0, 4.0, 5.0, 6.0), and 0.2*M* sodium phosphate (pH 6.0, 7.0), and 0.6 mL of 30% H_2O2 was added to each gel suspension while shaking. New aliquots of the H_2O_2 solution were added after 30, 90, and 150 min (0.73 mL each). The reaction was then continued for a total period of 24 h.

2. The effect of hydrogen peroxide concentration was studied in the range 1.0–6.5% final concentration. The incubations were run for 24 h at 22°C in 0.2*M* sodium acetate, pH 5.0.
3. The activation of thiol-agarose as a function of incubation time was studied with 3.5% H_2O_2 at pH 5.0.

Other matrices used as supports for thiolsulfonate groups. Beaded cellulose, Sephadex G-75, and Eupergit C were thiolated as beaded agarose was and then oxidized according to the standard procedure.

Kinetics of glutathione binding as a function of pH. TS-gel aliquots (2.5 g packed gel, containing 10 μmol reactive groups/mL) were equilibrated in conical centrifuge tubes at pH 3.5, 5.0 (0.1*M* sodium acetate), 6.0, 7.0, 8.0, and 8.5 (0.1*M* sodium phosphate). The reaction was started by mixing the gel samples with 3.0 mL of 15 m*M* glutathione solution adjusted to the corresponding pH. The suspensions were then vortexed. At different periods, 50-μL aliquots of supernatant were transferred from each suspension to 3.0 mL of 0.25 m*M* 2-PDS in 0.1*M* sodium phosphate, pH 8.0. The absorbance of the formed solution was measured at 343 nm and plotted as a function of incubation time. The rate of glutathione consumption was then determined from these plots as $t^{1/2}$ (the time required to decrease the amount of reactive structures by 50%). Controls for spontaneous oxidation of glutathione at each pH were run using 2.5 mL of buffer instead of gel. Blank experiments were performed at pH 5.0 and 8.0 using 2.5 g of Sepharose 4B instead of TS.gel.

Thiolation of BSA and sweet-potato β-amylase. This was performed by the heterobifunctional reagent *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) according to the method of Carlsson et al. (*14*). BSA dissolved in 0.1*M* sodium phosphate, pH 8.0, was reacted with 5× molar excess of SPDP (dissolved in ethanol). After changing to 0.2*M* sodium acetate, pH 4.5, by gel filtration on PD-10 columns, reduction with 25 m*M* DTT was performed. A thiol content of 4.0 mol SH groups/mol BSA was found. Sweet-potato β-amylase in 0.1*M* sodium phosphate, pH 6.8, was reacted with 30× molar excess of SPDP. After 30 min 1.5× molar excess (based on SPDP added) of DTT was added and reduction was run for 30 min. The thiolated β-amylase was then separated from excess DTT and other low-mol-wt reagents on a Sephadex G-25 column (PD-10). This procedure led to a thiol content of 5 mol/mol of β-amylase.

Analytical Methods

Titration of solid-phase bound thiolsulfonate groups. Three methods were used for quantification of the reactive structures generated by hydrogen peroxide oxidation of thiol-agarose:

1. The glutathione method: 2.5–3.0 g of suction-dried TS gels (disulfide gel as control) were equilibrated in 0.1*M* sodium phosphate, pH 7.0, in centrifuge tubes. Glutathione (15 m*M*,

3.0 mL) in the same buffer was added to each gel while mixing (vortex). The suspensions were incubated for 30 min at 22°C with mixing every 5 min. After centrifugation, 50-μL aliquots of supernatants were mixed with 3.0 mL of 0.25 m*M* 2-PDS in 0.1*M* sodium phosphate, pH 8.0. The absorbance at 343 nm was 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 the gels were then determined after drying for 24 h over phosphorus pentoxide in the dessicator. The amounts of glutathione that bound to the oxidized gels were calculated from the difference in absorbance readings at 343 nm between the glutathione blank and the gel supernatants.

2. More accurate determinations of the bound glutathione were performed by total amino acid analysis after thorough washing and drying of the gel derivatives.
3. The 2-thiopyridone method: TS and disulfide gels (aliquots of about 2 g of suction-dried gel each) were equilibrated on a glass filter funnel in 0.2*M* sodium acetate, pH 5.0, and suspended in 3 mL of the same buffer in glass vials. 2-Thiopyridone solution (0.5 mL, 50 mg/mL in methanol) was added while mixing (vortex). Incubation at 22°C was performed for 16 h with sporadic mixing. After exhaustive washing until free of unbound reagent, the gel was treated with 10 mL of 50 m*M* DTT in 0.1*M* sodium phosphate, pH 8.0. The 2-thiopyridone released was spectrophotometrically determined at 343 nm using an extinction coefficient of 8.02×10^3 (*14*). After determining the dry weight as explained above, the amount of 2-thiopyridone bound by the TS and disulfide gels was calculated as micromoles per gram of dry weight.

Amino acid analysis. The amino acid, peptide, or protein contents of the gel derivatives (previously dried over phosphorus pentoxide) were determined by total amino acid analysis after hydrolysis in 6*M* HCl for 24 h at 110°C.

Sulfur analyses. These were performed according to standard procedures.

Titration of sulfinic acid groups on the agarose derivatives. This was based on the method reported by Barnard et al. (*15*) for the estimation of soluble thiol-sulfonates. Two aliquots, about 12 g each, of suction-dried TS gel were washed in distilled water. One aliquot was kept as reference and the other was reduced with 100 m*M* β-mercaptoethanol in 0.1*M* sodium phosphate, pH 8.0 (100 mL) for 1 h during shaking. The reduced TS gel was washed with distilled water until free of reagents. Both reference and reduced TS gel aliquots were equilibrated with and suspended in 20 mL of 95% ethanol. A few drops of bromophenol blue solution (0.1%) were

added and titration performed with 0.025*M* NaOH. The number of micromoles of sulfinic acid groups formed as a result of the reduction was then calculated from the difference in amount of sodium hydroxide consumed by the two gel aliquots.

Thiol-group analyses. Thiol content of both soluble and insoluble material was determined spectrophotometrically by titration with 2,2'-dipyridyldisulfide in 0.1*M* sodium phosphate, pH 8.0, according to the method of Brocklehurst et al. (2).

Stability of gel-bound TS groups. Aliquots (2.0 g) of suction-dried TS gel were equilibrated and suspended in 3.0 mL of 0.1*M* sodium acetate, pH 5.0. Incubation was performed at two temperatures (4 and 30°C). Periodically (every seventh day) one aliquot of each incubation was titrated by the glutathione procedure. The total amount of reactive TS groups was then determined as a function of incubation time and temperature. Freeze-dried samples were stored at 4°C for up to 2 yr and the remaining reactive structures were determined after reconstitution of the gels as a function of time.

Stability as a function of pH: 2.0-g aliquots of suction-dried TS gel were equilibrated and incubated at 4°C for 72 h in the following buffers: 0.1*M* sodium acetate, pH 3.0, 4.0, and 5.0; 0.1*M* sodium phosphate, pH 6.0, 7.0, 8.0, and 8.5; and 0.35*M* ammonium carbonate, pH 9.0. After the incubation period, all the gel aliquots were reequilibrated in 0.1*M* sodium phosphate, pH 7.0, and residual reactivity toward glutathione was assayed.

Immobilization and Release of Ligands

Immobilization of thiolproteins. Thiolated BSA (10 mg/mL), thiolated sweet-potato β-amylase (1.9 mg/mL), β-galactosidase (1.4–4.0 mg/mL), urease (1.9 mg/mL), alcohol dehydrogenasee (1.1 mg/mL), and papain (1.2 mg/mL), (each dissolved in 0.1*M* sodium phosphate, pH 7.0 [5.0 mL]), were incubated in plastic tubes with screw caps with 2.0-g suction-dried aliquots of TS gel (250 μmol reactive groups/g dried gel) and slowly rotated end-over-end at room temperature for 16 h. After the incubation, the insoluble derivatives were thoroughly washed with coupling buffer, 1*M* NaCl, and the assay buffer (as indicated below), in which the gel derivatives were stored until use. Samples from the different gel derivatives were washed with distilled water, dried over P_2O_5, and subjected to amino acid analysis.

Determination of activities of immobilized enzymes. This was performed according to the following methods:

1. Determination of β-amylase activity was performed with 1% soluble starch in 0.05*M* sodium acetate, pH 4.8, according to the method of Bernfeld (*16*).

2. β-galactosidase activity was assayed in 0.1*M* potassium phosphate, pH 7.5, using *o*-nitrophenyl β-D-galactopyranoside (ONPG) as substrate (*17*).
3. Ureolytic activity determination was performed on 0.2*M* urea in 0.2*M* TRIS/HCl buffer, pH 7.2, containing 1 m*M* EDTA (*3*).
4. Alcohol dehydrogenase activity was determined with 0.1*M* sodium pyrophosphate, pH 8.8, containing 5 m*M* NAD^+ and 0.3*M* ethanol (*18*).
5. Papain activity was determined with the synthetic substrate *N*-benzoyl-L-arginine ethyl ester (BAEE) (*19*).

Binding of thiolated BSA by thiolsulfonate agarose. TS gels of different activation levels (63, 157, and 250 μmol reactive groups/g dried gel, respectively, according to the glutathione titration procedure) were equilibrated with 0.1*M* sodium phosphate, pH 7.0, in 10-mm-id columns (3.0-mL packed beds). Thiolated BSA in the same buffer, 4.2 mg/mL (containing 4.0 mol of SH groups/mole) was pumped through a 25 mL/h and the effluents from the chromatographic columns monitored continuously at 280 nm with a Pharmacia UV-1 detector coupled to a Pharmacia Rec 2 recorder. After saturation (when protein concentration in eluates was the same as that of the feeding solution), the beds were thoroughly washed with 0.5*M* NaCl in the sample buffer; 0.2*M* sodium acetate, pH 4.0; 6*M* urea; and water. After removing the different gel derivatives from the columns and drying them, their protein content was determined by amino acid analysis (*see above*).

Release of gel-bound peptides and proteins by reductive cleavage. This was performed by incubating the gel derivatives (2–3 g of suction-dried material) with 10 mL of 100 m*M* DTT in 0.1*M* sodium phosphate, pH 8.0, under end-over-end rotation for 1 h. Then the gel derivatives were thoroughly washed with buffer, 1*M* NaCl, 6*M* urea, and water. The remaining peptide or protein bound to the gel derivative was then determined by total amino acid analysis as described above. The reduction could also be performed with other low-mol-wt thiols, such as β-mercaptoethanol and cysteamine, using a concentration of 0.2*M* and a pH of 8.5–9.0. If the gel is to be regenerated, however, it is recommended always to perform a final treatment with 5 m*M* DTT at pH 8.0 to remove possible mixed disulfides.

Reaction of thiolsulfonate gel with different low-mol-wt substances. TS-gel aliquots (2.5 g, suction-dried) were incubated for 24 h at 22°C during end-over-end rotation with 10 mL of the following agents: 6*M* guanidine HCl, 6*M* urea, 1*M* NaCl, 0.1*M* KI (all in 0.1*M* sodium phosphate, pH 7.0). Incubation with sodium azide (0.1% in 0.1*M* sodium phosphate, pH 7.0) and 0.1*M* Tris, pH 7.2, was performed during 1 wk at 22°C. After incubation, all gel aliquots were washed with 0.1*M* phosphate, pH 7.0, and the remaining reactive groups determined by titration with glutathione.

Reaction of TS gel with nonthiol amino acids and peptides compared with cysteine. TS-gel aliquots (2.5 g, suction-dried) equilibrated in 0.1*M* sodium phosphate, pH 7.0, were incubated with

1. 3.0 mL of 15 m*M* cysteine;
2. 3.0 mL of 15 m*M* glycine;
3. 3.0 mL of 100 m*M* glycylglycine;
4. 10 mL of an amino acid mixture (2.1 m*M* in each amino acid), glutamic acid, methionine, histidine, arginine, lysine, serine, tyrosine, phenylalanine, and proline.

All substances were dissolved in 0.1*M* sodium phosphate, pH 7.0. After incubation for 16 h at 22°C with end-over-end rotation, all the gel aliquots were thoroughly washed, were dried over P_2O_5, and amino acid analysis was performed.

Reactivation of used gel. This was performed by H_2O_2 oxidation at acidic pH according to the standard procedure described above, after release of bound material and thorough washing of the gel. The maximum number of reactivations for a thiolsulfonate-agarose derivative originally containing 407 μmol active groups/g dried material was established by the following set of experiments: 30 g (suction-dried) thiolsulfonate gel was reduced with 50 m*M* DTT in 0.1*M* sodium phosphate, pH 8.0, for 2 h during shaking. After washing the gel thoroughly, it was oxidized with H_2O_2 as described above. This procedure was repeated. Reactivity towards glutathione and binding capacity for thiolated BSA was determined after each regeneration. Finally the thiolsulfonate gel after the second regeneration was reduced, thoroughly washed, and converted into a pyridyldisulfide gel derivative by incubation with 2-PDS according to the method of Axén et al. (*13*).

RESULTS

Preparation of activated gel by oxidation of thiol-agarose. When agarose derivatives containing free thiol groups were treated with hydrogen peroxide at acidic pH for 10–30 h at room temperature, the gel derivatives acquired the property of binding large amounts of thiol compounds of both low- and high-mol-wt type (Table 1). On the other hand, the disulfide-containing gel formed by air oxidation at alkaline pH of the same thiol-agarose derivatives only bound trace amounts of thiols when incubated with the latter compounds under similar conditions (Table 1).

To examine the nature of the activated structures formed by the H_2O_2 oxidation and the mechanism for the coupling, the reaction of the activated gel with one of the thiol compounds, namely reduced glutathione, was studied in more detail. The disappearance of reduced glutathione from the liquid phase, after being added to suspensions of the activated gel, was

Table 1
Thiolpeptides and Proteins Immobilized on TS-Agarose and Disulfide-Agarose, Before and After DTT Treatment

Gel derivative	Thiol compound	Amount bound, mg/g dried derivative	Amount remaining after DTT treatment, mg/g dried derivative
TS-agarose	Glutathione	51.9	0.0
	Thiolated BSA	302.1	33.2
	β-Galactosidase	134.4	12.0
Disulfide agarose	Gluthathione	3.0	-
	Thiolated BSA	13.3	-
	β-Galactosidase	7.1	-

followed by back-titration with the thiol-titrating reagent 2,2'-dipyridyldisulfide (2-PDS) of free thiol groups remaining in solution. In a typical experiment with an activated gel prepared from a thiol-agarose derivative originally containing about 20 μmol thiol groups/mL, 10 μmol glutathione/mL gel was consumed. The agarose derivative contained 51.9 mg glutathione/g dried gel (equivalent to 10.6 μmol/mL swollen gel) as determined by amino acid analysis. The glutathione could not be removed from the gel by extensive washing with alkaline or acidic buffers of various ionic strengths or with detergents. However, it was released by treatment of the gel with an excess of DTT (Table 1). Experiments with other low-mol-wt thiols gave similar results. Also the thiol-thione tautomery-stabilized 2-thiopyridone reacted quantitatively with the activated gel at pH 5 and could subsequently be released by DTT treatment.

Neither the gel oxidized by hydrogen peroxide nor the glutathione-agarose derivative obtained when this gel was reacted with glutathione contained any detectable amount of free thiol groups. However, after treatment of the glutathione-agarose derivative with a large excess of DTT (100 m*M*) and careful washing at acidic pH to remove excess DTT and avoid reoxidation, a thiol content of 260 μmol/g dried gel was obtained (Table 2). This figure represents 54% of the thiol content of the original thiol-agarose derivative. Elemental analysis proved the activated gel to have the same sulfur content as the original thiol-agarose, and that this amount increased by about 50% as a result of the immobilization of glutathione (Table 2). Thus no sulfur-containing compound was released as the result of the immobilization of the thiol. When glutathione was removed from the gel derivative with a large excess of DDT, the sulfur content of the gel decreased to that of the activated gel. Acid–base titration of the activated gel before and after treatment with mercaptoethanol indicated the formation of an acidic group with a $pK_a < 2$ as a result of the coupling of the thiol.

Table 2
Sulfur and Thiol Content of Different Agarose Derivatives

Gel derivative	S, %	SH groups, μmol/g derivative
Thiol-agarose	1.6	480
TS-agarose	1.6	negligible
Glutathione-agarose[a]	2.3	negligible
Glutathione-agarose, DTT treated	1.6	260

[a]Prepared by coupling glutathione to TS-agarose.

These results are in agreement with the formation of thiolsulfonate on the gel by oxidation and subsequent formation of a gel-bound sulfinate group when the gel reacts with glutathione. These are further outlined in the discussion section.

Optimization of the activation procedure. Using thiol-agarose derivatives containing 60–800 μmol thiol groups/g dried derivative, activated gels with a capacity to bind 30–400 μmol low-mol-wt thiols/gram dried gel have been prepared (in the three-step thiolation procedure described above, the thiol content of the gel is determined by the amount of eip-chlorohydrin used in the initial step).

The activation procedure was optimized for a thiol-agarose derivative containing 430 μmol SH group/g dried derivative by studying the capacity of the formed disulfide oxide-agarose derivatives to bind glutathione as a function of different experimental conditions. The pH optimum for the activation was found to be in the range 4–5. As pH was increased above 5 and approached neutrality, the degree of activation decreased rather drastically. More acidic pH was also less efficient (*see* Fig. 1). A final concentration of 3.5% H_2O_2 in the reaction mixture was found to be optimal, although the reaction worked with 1–6.5% H_2O_2.

Figure 2 shows the oxidation of the thiol-agarose derivative containing 430 μmol thiol groups/g dried gel as a function of incubation time at room temperature, pH 5, and a hydrogen peroxide concentration of 3.5%. Under these conditions the optimal degree of activation (almost 215 μmol reactive groups/g dried derivative) was reached after 30 h of continuous shaking.

Stability of the activated gel. The stability of the activated gel when stored under different conditions was examined by its ability to bind glutathione. After incubation of the gel as a suspension in 0.2*M* sodium acetate, pH 5.0, at 4°C for 2 mo and at 30°C for 1 mo, no decrease in reactivity could be detected. When the gel was stored in lyophilized form at 4°C, 100% of the reactivity was conserved after 6 mo and 71% after 2 yr. The gel-bound thiolsulfonate groups were found to be very stable in a wide pH range (pH 3–8) (Fig. 1). However, at more alkaline pH a significant decrease in

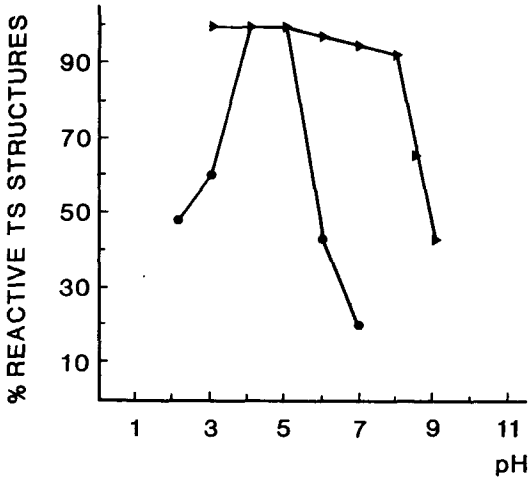

Fig. 1. The effects of pH on the formation and stability of thiol-reactive agarose-bound structures. (i) Influence of pH on the formation of reactive structures by oxidation of thiol-agarose with 3.5% hydrogen peroxide for 24 h (-●-●-●-●-). (ii) Stability of agarose-bound thiolsulfonate structures as a function of pH (-▲-▲-▲-▲-). TS-gel aliquots were incubated for 72 h at the corresponding pH at 4°C, then reequilibrated at pH 7 and residual reactivity towards glutathione determined. Reactivities are expressed as percentages of the values obtained at pH 5.

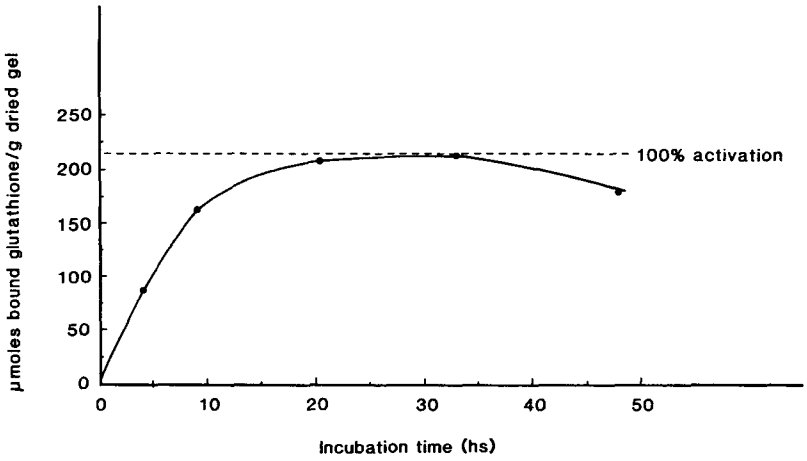

Fig. 2. Formation of reactive structures as a function of incubation time. Thiol-agarose (430 µmol of SH groups/g of dried gel) was incubated with 3.5% hydrogen peroxide in 0.2*M* sodium acetate, pH 5.0, at room temperature. At selected periods, samples were withdrawn, filtered and washed on sintered glass filters and binding of glutathione determined. In accordance with the proposed mechanism for the activation reaction based on the different analyses, the maximal number of thiolsulfonate groups (100% activation) should be 50% of the original thiol content.

Table 3
Kinetics of the Glutathione Reaction
with TS-Agarose as a Function of pH

Buffer, 0.1*M*	pH	$t^{1/2}$, min
Sodium acetate	3.5	–[a]
Sodium acetate	5.0	26
Sodium phosphate	6.0	18
Sodium phosphate	7.0	2
Sodium phosphate	8.0	<2
Sodium phosphate	8.5	<2

[a]No reaction detected within 2-h incubation.

stability was observed; e.g., at pH 9 >50% of the reactive groups were lost after 72 h of incubation. The gel-bound thiolsulfonate groups were also compatible with sodium azide and Tris. Thus, the activated agarose gel showed almost full reactivity (95% of original) after incubation with 0.1% NaN_3 in 0.1*M* sodium phosphate, pH 7.0, and 0.1*M* Tris buffer, pH 7.2, for 1 wk at room temperature. Compatibility was also demonstrated for 6*M* guanidine-HCl and 6*M* urea at pH 7.

Immobilization and Release of Ligands

Immobilization of low-mol-wt compounds, kinetics. The effect of pH on the rate of the reaction between the thiolsulfonate groups on an activated agarose containing 250 μmol reactive groups/g dried derivative (equal to 10 μmol active groups/mL packed gel) and glutathione was studied in the range 3.5–8.5, using an initial twofold molar excess of glutathione. The half-life ($t^{1/2}$) for the gel-bound active groups was determined for each pH value. Smaller $t^{1/2}$ means higher rate of reaction.

Table 3 shows that no reaction can be detected at pH 3.5. At pH 5 immobilization occurs at a reasonable rate. The rate increases dramatically when the pH is increased to neutral and weakly alkaline and becomes too fast to follow with the described method at pH ≥ 8.

Immobilization of thiolproteins. The enzymes β-galactosidase from *E. coli*, urease from jack bean, and alcohol dehydrogenase from baker's yeast, all three containing exposed thiol groups, were immobilized to thiolsulfonate-agarose with 250 μmol activated groups/g dried derivative. Depending on the amount of enzyme used in the coupling reaction, derivatives with 20–200 mg protein/g dried derivative could be prepared. For the low-load derivatives, about 80–90% of the added protein was immobilized (Table 4). Assay of the low-load derivatives showed that 50–70% of the applied activity was conserved after immobilization. The thiolprotease papain was also immobilized, however, with loss of the activity.

Table 4
Reversible Immobilization of Enzymes on TS-Agarose[a]

Enzyme	Immobilization yield, %	Dried derivative, mg/g	Enzymatic activity of derivative
β-Galactosidase *E. coli*	90	53.8	Active
Alcohol dehydrogenase Bakers' yeast	85	39.7	Active
Urease Jack beans	84	67.7	Active
Papain Carica papaya	90	44.8	Inactive
β-Amylase (thiolated) Sweet potato	80	63.6	Active

[a]Amounts of enzyme corresponding to 20–25% of the binding capacity of the gel were used.

BSA did not bind to the activated agarose in spite of a large excess of albumin, use of high-capacity gel, and long periods of incubation. Immobilization, however, occurred if the reaction was performed in the presence of 6*M* guanidine-HCl. After introduction of 4 mol *de novo* thiol groups/mol of serum albumin by a two-step procedure involving SPDP and DTT, it was possibile to bind large amounts (more than 300 mg/g) of BSA to the activated gel (Table 1).

Another protein that has been immobilized after thiolation is sweet-potato β-amylase. Preliminary experiments showed that this enzyme also was active after immobilization. More details about the properties of this derivative will be reported elsewhere.

To examine the importance of the degree of activation of the thiosulfonate-agarose for the capacity of binding high-mol-wt thiols, thiolated BSA (4.0 mol SH groups/mol protein) solutions were pumped through columns with activated gels with varying content of thiolsulfonate groups until the gels were saturated. The amount of immobilized BSA was determined by amino acid analysis of the dried derivatives. Table 5 shows the albumin-binding capacities for three degrees of activation (glutathione-binding capacity).

Release of the immobilized proteins, specificity of binding. Most of the bound albumin and β-galactosidase could be removed from the agarose derivatives when treated with 100 m*M* DTT at pH 8 for 1 h (Table 1). However, even after extensive washing of the reduced gel derivatives with buffers of different pH with varying ionic strength and denaturing agents, such as 6*M* urea, about 10% of the originally bound proteins remained on the gels (Table 1).

Table 5
Binding Capacity for Thiolated BSA
of TS-Gels with Different Activation Levels[a]

μmol TS-groups/g dried derivative[b]	mg protein/g dried derivative[c]
63	88.1
157	216.7
250	301.4

[a]The BSA used had an average of 4.0 mol of SH groups/mol.
[b]Glutathione titration.
[c]Amino acid analysis.

To see if sulfenamide or other covalent bonds besides disulfide bonds can be formed during the conditions used for immobilization, the amino acids glycine and cysteine, the dipeptide glycylglycine, and an amino acid mixture composed of glutamic acid, methionine, histidine, lysine, arginine, serine, tyrosine, phenylalanine, and proline were incubated with samples of activated agarose at pH 7 and room temperature for 16 h. Amino acid analysis of the thoroughly washed gels showed only trace amounts (<1 μmol/g dried gel) in all cases except for the one reacted with the thiolamino acid cysteine, in which 130.5 μmol amino acid/g dried g remained.

Reactivation of used gel. After the thiol ligands have been released from the gel by a thioldisulfide exchange reaction with an excess of low-mol-wt thiol, the gel contains immobilized sulfinate and thiol groups (*see* Fig. 3, Discussion). It was found that these gel-bound thiol groups could be converted into new thiolsulfonate groups with the same treatment as was used to activate the original thiol gel, i.e., by H_2O_2 oxidation at acidic pH. When a reduced gel derivative originally containing 407 μmol of thiolsulfonate groups was reoxidized this way, 192 μmol of thiolsulfonate groups were formed. After treatment of the reactivated gel with an excess of the low-mol-wt thiol DTT it could again be partly regenerated as described above, but this time the thiolsulfonate content decreased further, to 104 μmol/g dried derivative.

DISCUSSION

Structure of Activated Gel and Mechanism of Reaction

The results indicate that glutathione, 2-thiopyridone, and the other thiol compounds are bound to the H_2O_2-treated agarose by disulfide bonds. That the binding is not obtained via a simple thiol–disulfide exchange reaction is obvious from the fact that only trace amounts of thiols

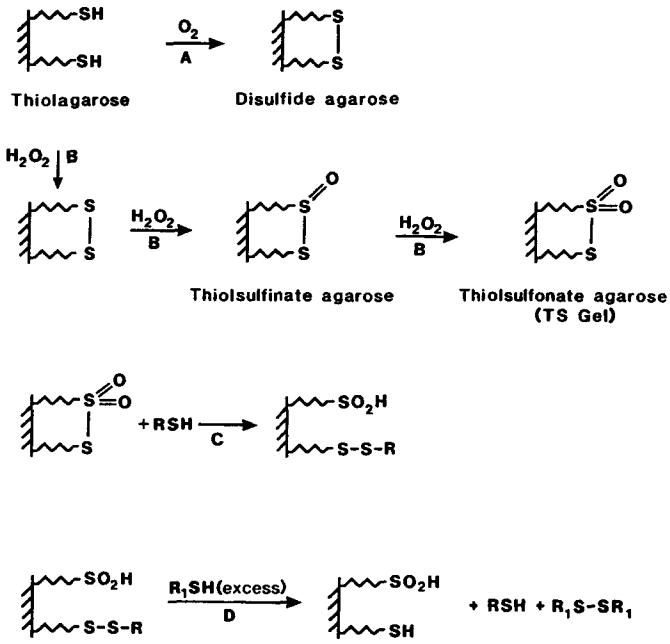

Fig. 3. Proposed structures and reactions of disulfide and TS-gels. **A:** Formation of alifatic disulfides by air oxidation (control gel). Thiol-agarose was incubated in 0.1*M* sodium phosphate, pH 8.0, containing traces of Cu^{+2}. Air was bubbled through the suspension for 6 h. **B:** Conversion of agarose-bound thiol groups to thiolsulfonate groups by oxidation with hydrogen peroxide (3.5%) in 0.2*M* sodium acetate, pH 5.0. **C:** Immobilization of alifatic thiols by their reaction with agarose-bound thiolsulfonate groups. **D:** Release of gel-bound ligand by reaction with excess of a low-mol-wt thiol (e.g., 100 m*M* DTT in 0.1*M* sodium phosphate, pH 8.0).

bound to the disulfide-containing agarose (control experiment). These results also agree with the well-known poor reactivity of alifatic thiols with alifatic disulfides at acidic pH.

A likely candidate for a reactive group formed in the agarose is a disulfide oxide structure (*12*). Disulfide oxides with both one and two oxygen atoms, thiolsulfinates, and thiolsulfonates, respectively, are known to be formed subsequently to the initially formed disulfides when low-mol-wt thiols are treated for long periods with oxidation agents as H_2O_2 and peracetic acid (*7*). Both these groups, in spite of being relatively stable, react vigorously with alifatic thiols at both acidic and alkaline pH. Based on the results presented above, in particular the thiol and sulfur analysis of the agarose derivatives after H_2O_2 treatment, coupling, and release of bound low-mol-wt thiols, we propose a mechanism for activation of thiol-agarose and coupling of and release of thiol compounds as outlined in Fig. 3. Under the described conditions the major reactive group generated most probably is thiolsulfonate rather than thiolsulfinate, since each cycle of

binding, release, and regeneration leads to a 50% decrease in the binding capacity and thiol content. The sulfinic acid residues formed from thiolsulfonates cannot be reduced back to thiols when treated with an excess of low-mol-wt thiols, contrary to the sulfinic acid groups formed with thiolsulfonates (*19*), which under the same conditions are easily converted to thiols. A further support for thiolsulfonate as reactive groups is the occurrence of an acidic group with a $pK_a < 2$ after release of the bound thiol from the agarose. This group might well be a bound sulfinic acid since, according to the literature, sulfinic acids have pK_a of about 1.5 (*15*). However, it is reasonable to assume that, as with the low-mol-wt thiols, the oxidation to thiolsulfonate proceeds via alifatic disulfide and thiolsulfinate (Fig. 3B).

Even if the experimental results indicate that the majority of active groups in the final product are thiolsulfonates, the presence of minor amounts of other oxidation stages of sulfur, such as in thiolsulfinates and sulfonic acid groups, cannot be excluded. Thus oxidation of thiol-agarose gel for extended periods of time (>30 h) gave derivatives with less capacity of binding glutathione than derivatives obtained with the optimal time. Acid–base titration of such overoxidized gels also showed presence of significant amounts of acidic groups at low $pK_a < 2$, which might be sulfonic acid groups. The number of reactive groups formed when using optimal conditions, i.e., pH 5, 3.5% H_2O_2, and a 30-h reaction time at room temperature, is almost 100% of the theoretical maximum value. Provided that the proposed mechanism for activation and coupling is valid, this corresponds to 215 μmol/g dried gel, that is, 50% of 430 μmol/g dried derivative (the original thiol content of the agarose derivative.) Preliminary experiments have shown that the optimal H_2O_2 concentration and reaction time are interdependent and somewhat dependent on the thiol content of the original thiol-agarose.

Immobilization and Release of Thiol Compounds

The pH/rate dependency for the reaction of the agarose-bound thiolsulfonate groups with glutathione is similar to the ones obtained for most thiol–disulfide exchange and alkylation reactions and is because the unprotonated form of the thiol (thiolate ion) is a better S-nucleophile than the protonated form. Because of the high pK_a values of most alifatic thiols, the concentration of the thiolate ions increases with pH and thus also the rate of immobilization. Certain protein thiols sometimes do not follow this simple ionization pattern because of microenvironmental effects. For example, the papain thiol reacts with 2,2′-dipyridyldisulfide at pH 4 about 15 times faster than at pH 8 (*20*).

The thiolsulfonate agarose derivatives proved to be excellent for immobilization of thiol-containing proteins, as demonstrated with β-galactosidase, urease, alcohol dehydrogenase, and papain. The observed decrease

in activity for the first three enzymes most probably is only apparent and attributable to diffusion control, which usually is the case for enzymes immobilized on solid phases. In the case of papain all activity was lost, since its only free SH group also is essential for activity. Preliminary experiments have shown that most of the activity will be regained when the enzymes are released from the agarose by treatment with DTT.

BSA, although it contains 20–60% mercaptalbumin with a free thiol group, did not bind. This is probably because the mercaptoalbumin thiol is located in a hydrophobic pocket and thus, for sterical and polarity reasons, is not available for reaction with the gel-bound hydrophilic disulfide oxide group. The immobilization did occur as expected in presence of 6*M* guanidine-HCl, in which milieu albumin is denaturated and the thiol group exposed.

Proteins with inaccessible thiol groups or nonthiol protein can also be immobilized with the present method, as shown with BSA and sweet-potato β-amylase after providing them with *de novo* thiols.

The amounts of proteins immobilized by this method (Table 1, 4) are in the same range as previously reported for the established techniques (*21*). As can be seen in Table 5, there is a good correlation between degree of activation of the gel derivatives and the albumin-binding capacity. At each activation level about 2% of the activated groups seem to be utilized for the coupling. The remaining thiolsulfonate groups could be titrated with glutathione after the immobilization of albumin was completed. In earlier studies with other coupling methods, we have found that 300–400 mg protein/g dried derivative is the maximum amount of BSA (and other proteins) that can be bound to agarose (J. Carlsson, unpublished results). The limiting factor most probably is the space available for large molecules, rather than the number of reactive groups. To obtain protein-binding capacities approaching these figures, around 250 μmol reactive groups/g of dried gel needs to be introduced into the agarose.

According to the proposed binding mechanism (Fig. 3) proteins are bound to the solid phase by disulfide bonds. It should therefore be possible to release them by reduction or a thioldisulfide exchange reaction involving a large excess of a low-mol-wt thiol. However, about 10% of the originally bound protein remained on the gels even after extensive washing at different pH values and with denaturing agents (Table 1). No glutathione could be detected on the gel after similar treatment of glutathione-agarose gel. The residual protein might be bound to the gel by strong multipoint noncovalent interactions, which are less likely to occur with the much smaller glutathione molecule, which is only a tripeptide. Another possibility might be that the gel-bound thiolsulfonate groups are not completely specific for thiol groups, but also to a certain extent react with other amino acid side-chain functional groups. Thus low-mol-wt thiolsulfonates react with amines under formation of stable sulfenamides in some conditions (22). However, under the conditions outlined here,

Table 6
Regeneration of Agarose-Bound Thiolsulfonate Groups

Gel derivative	μmol reactive groups/g dried derivative[a]	Binding capacity for thiolated BSA, mg/g dried derivative
TS-agarose	407	308.5
TS-agarose, first regeneration	192	210.2
TS-agarose, second regeneration	104	107.4

[a]Glutathione titration.

only trace amounts of nonthiol amino acids and peptides were bound to the thiolsulfonate agarose. The figures in Table 6 agree well with the proposed mechanism for activation, binding, and release outlined in Fig. 3.

This suggests that every cycle of activation, binding, release, and reactivation leads to a decrease of the thiolsulfonate group content by 50%, as 50% of the thiols are turned into gel-bound sulfinate groups. The binding capacity for original and regenerated TS gels (Table 6) agrees well with what was found for activated gels originally provided with the corresponding content of activated groups (Table 5).

Depending on the requirement of the activated gels' ligand-binding capacity, it thus seems reasonable to regenerate the gel at least twice. Provided that the original gel contains 800–1000 μmol SH groups/g dried derivative, the binding capacity for low-mol-wt ligands will still be 100–150 μmol/g dried gel after the second regeneration. Another way of reusing the gel derivative after release of bound material is to convert the gel-bound thiol groups to reactive disulfides by reaction with 2.2'-dipyridyldisulfide (*1*). The thiol gel formed by reduction of the activated gel after the second regeneration thus was turned into a reactive disulfide derivative containing 42 μmol 2-pyridyldisulfide groups/g dried gel. Such a regeneration, unlike the oxidative procedure, can be repeated over and over again with a preserved degree of activation and, thus, ligand-binding capacity.

Comparison of Thiolsulfonate and 2-Pyridyldisulfide as Active Groups on Solid Phases

The thiolsulfonate-agarose derivative has the same capacity to bind low- and high-mol-wt thiol ligands as the previously described 2-pyridyldisulfide agarose derivatives (Thiopropyl Sepharose 6B and Activated Thiol Sepharose 4B, Pharmacia) (*1*). The method for introducing the thiolsulfonate groups, oxidation of thiol-agarose with H_2O_2 at acidic pH, is a cheap and simple method that also has proven to be very reproducible. Since the oxidation goes to completion, the degree of activation can easily be

adjusted by using agarose with different thiol contents. The gel-bound thiolsulfonate groups seems to be very stable under various conditions of pH and the like. Unlike the 2-pyridyldisulfide group, it is unaffected by sodium azide, which often is used to prevent bacterial growth in gel suspensions.

In the immobilization step there is no release of any low-mol-wt component, as the sulfinic acid groups formed are gel-bound. In contrast to immobilization on 2-pyridyldisulfide agarose, in which 2-thiopyridone is released as a result of the coupling reaction, the course of the reaction cannot be easily monitored. On the other hand, this feature is an advantage in applications in which the activated gel is used to eliminate thiol molecules from a solution in which the nonbound components are of interest, since no contamination occurs.

The pyridyldisulfide gels bind virtually to all thiol compounds capable of reaction with low-mol-wt thiol-titrating reagents in solution. At high ionic strength they have also been found to bind nonthiol proteins, especially immunoglobulins, though a noncovalent interaction—a property that recently has been utilized in the so-called thiophilic adsorption chromatography (*23*). The thiolsulfonate agarose derivatives do not show thiophilic adsorption properties, and also seem more specific in their interaction with thiol compounds. Mercaptoalbumin in native form, e.g., which has been purified by covalent chromatography on 2-pyridyldisulfide agarose (*24*), does not bind at all to thiolsulfonate-agarose. In spite of the fact that the thiolsulfonate gels can be regenerated only a few times, the economies might still be better than can be obtained with gels containing reactive disulfide groups, since the chemicals needed for the preparation of the former gel are much cheaper.

Preliminary studies have proven that thiolsulfonate groups can be introduced by the present method into other solid-phase material also, such as cellulose, crosslinked dextran (Sephadex), and polyacrylamide, provided that the materials can be thiolated. The described thiolsulfonate-agarose and other thiolsulfonate-substituted solid phases are interesting alternatives to the pyridyldisulfide containing solid phases for covalent chromatography and reversible immobilization of thiol substances.

ACKNOWLEDGMENTS

We are grateful to IPICS, Uppsala University, Sweden; PEDECIBA (Programa para el desarrollo de las Ciencias Básicas) and the "Programas de Biotechnologia e la Universidad de la República," Montevideo, Uruguay, for financial support. We also thank Anna-Tora Martin and Erik Ringberg for valuable suggestions and skillful technical assistance, Gull-Marie Hedén for excellent secretarial assistance and Lars Rydén for helpful comments on the manuscript.

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