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Preparation of chromatographic matrices by free radical addition ligand attachment to allyl groups

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Abstract

Chromatographic matrices were produced by free radical addition reactions between immobilised allyl groups and suitable ligands. Efficient addition and high ligand densities were readily obtained with mercaptoacids, glutathione, mercaptoethanol and sodium bisulphite, using aqueous solvation. Addition of nitrogen containing thiol ligands other than glutathione was also demonstrated, although heat or radiation catalysis was required. Addition of cysteamine or mercaptoacids resulted in spacer arm derivatives, suitable for further ligand attachment chemistry. Mercaptoacetic acid and bisulphite attachment was used to prepare cation-exchange matrices. Glutathione derivatives were compared with matrices prepared conventionally, for affinity chromatography of glutathione transferase. © 1998 Elsevier Science B.V.

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1. Introduction

In previous reports controlled matrix activation with allyl reagents and efficient ligand attachment to the brominated derivatives was reported [1,2]. High ligand densities were obtained easily, despite aqueous solvation. In particular, the high levels obtained on Perloza, a bead cellulose with good attributes for laboratory and industrial use [3–6], contrasted with low values obtained with conventional reagents [1]. Methods of allyl group modification other than aqueous bromination were unsuccessful. Because the allyl group is not a conjugated alkene, direct nucleophilic addition is not favoured. However, free radical methods have been used in solution for addition of thiols, aldehydes, phosphites and bisulphite to simple alkenes [7–10]. Free radical addition might therefore be used to attach ligands, containing one of these groups, to allyl matrices.

Thiols and the bisulphite ion, in particular, could be used to generate chromatographic matrices and/or intermediates for further ligand attachment chemistry. It was presumed that amino groups would not react by this chemistry [11]. Therefore specific attachment (thioetherification) of ligands containing both thiol and amine groups could be possible. Many thiol ligands are available and others can be prepared by simple, inexpensive methods [12]. The properties of radical addition reactions have been reviewed extensively [13–16]. Radical addition has been used to attach alkene ligands to thiol Sepharose, using irradiation [17] or peroxide/heat [18] catalysis.

In this report, facile addition of various organic thiols and bisulphite to allyl matrices is reported. Other additions, requiring catalytic heating and/or irradiation, are also described. Advantages of this

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chemistry over previous ligand attachment methods and possible applications are discussed.

2. Experimental

Thiolacetic acid, mercaptoacetic acid, thiosalicylic acid, cysteamine·HCl, mercaptopropionic acid and mercaptopropanediol were from Acros (Geel, Belgium); mercaptosuccinic acid and thiophenol from Aldrich (Milwaukee, WI, USA); reduced glutathione and equine glutathione transferase from Sigma (St. Louis, MO, USA); N-bromosuccinimide, ethanolamine, cysteine, 4-methoxyphenol, ammonium persulphate, benzoyl peroxide and 1-chloro-2,4-dinitrobenzene, from BDH (Dorset, UK); 2-mercaptoethanol and cysteine·HCl from Riedel-de Hahn (Seelze, Germany); sodium metabisulphite from Ajax Chemicals (Auburn, Australia); and sodium sulphite from May and Baker (Manchester, UK). Mercaptobutyric acid and mercaptohexanoic acid were prepared as described before [2]. The N-ethylamide of 3-mercaptopropionic acid was made from methyl 3-mercaptopropionate [19], by stirring with 25% ethylamine in methanol under nitrogen for 98 h at room temperature. All other chemicals were analytical grade.

Glutathione Sepharose was from Amrad Pharmacia (North Ryde, Australia). Allyl glycidyl ether (AGE) and allyl bromide (AB) activated matrices were prepared and titrated by the methods reported previously [1]. They are identified by the volume of allyl reagent used (ml/g suction-dried matrix), expressed as a percentage. Perloza 100 fine (80–100 μm beads) derivatives were used unless otherwise stated. Analyses of samples, oven-dried for 1.5 h at 110°C, were carried out by the Chemistry Department, University of Otago, Dunedin, New Zealand.

2.1. Ligand addition methods

Reactions were mixed by rotation (Ballmill roller or Cole-Parmer Roto-Torque rotator) or shaking (Ika Vibra-mix) at room temperature, or incubated in a water bath at 60–70°C, except for irradiated samples. These were irradiated with either a Tungsram 9043 Infrasatin IR 2 infrared lamp, placed 15–20 cm distant (with shaking for 3 h) or a 365 nm UV lamp (Cole-Parmer) placed 5 cm distant (1–5 h without mixing). Reactions were mixed in glass vials or pyrex beakers (irradiated samples), sealed with polyethylene.

2.1.1. Mercaptoacids and glutathione

Initially, addition (3–4 h, 60°C) of mercaptopropionic acid (100 μl) to allyl Perloza (1 g) included 2.5 mg ammonium persulphate and water (1–5 ml) for mixing. Variations were reaction at room temperature (16 h), exclusion of ammonium persulphate and inclusion of methoxyphenol (20 mg). The standard method for mercaptopropionic acid and mercaptoacetic acid (MAA) addition to activated and control matrices was a 4–8 h reaction at 60°C, without persulphate. Variations of MAA addition tested were temperature, reagent proportion and prior adjustment to pH 6 with 30% NaOH. Mercaptohexanoic acid (0.2 ml), mercaptobutyric acid (0.5 g), thiosalicylic acid (0.2 g) and N-ethyl mercaptopropionamide (0.2 g), dissolved in 1 ml of 95% ethanol or glacial acetic acid, were reacted with allyl Perloza (1 g) for 48 h at 60°C. Other mercaptobutyric acid samples (prepared likewise except 0.2 ml of ligand was used) were reacted by irradiation methods. Mercaptosuccinic acid (0.15 g) was mixed with allyl Perloza (1 g) and water (1 ml) for 16 h at 60°C or 64 h at room temperature. Glutathione (GSH) free acid was reacted likewise, but 0.2 g/g matrix was used. Another GSH addition (at 60°C) used 0.2 *M* phosphoric acid instead of water.

2.1.2. Mercaptoalcohols

Mercaptoethanol (100 μl) was mixed with AGE Perloza (1 g) at room temperature for 24–48 h or 60°C for 16 h. Mercaptopropanediol (200 μl) was reacted with AGE Perloza (1 g) for 72 h at room temperature. Afterwards, unreacted allyl groups were determined by back-titration with MAA [1] and the addition level calculated by difference.

2.1.3. Bisulphite

The initial reaction mixture was 0.1 g sodium sulphite, 0.1 g sodium metabisulphite and 5–10 ml water per g allyl Perloza. For addition at various pH values, a 10 molar excess of sodium metabisulphite or bisulphite (±1 ml of 4 *M* acetate), adjusted to the target pH (5–8) with 7.5 *M* NaOH or 3 *M* HCl was

used [20]. Improved AB Perloza reaction conditions used a solution (1 ml/g matrix) containing equal parts by weight of sodium metabisulphite and sodium sulphite, without extra water or acetate buffer. The total sulphite and bisulphite content was 32 or 40% [20]. This method was used for a control reaction with unmodified Perloza. Ultimately, equal parts of the salts were used without prior dissolution (plus 0.3–0.4 ml water/g Perloza to allow even mixing). This method was used with 14% AB (0.38 mmol/g) and 55% AGE Perloza (0.39 mmol/g) for molar excess variations (1–5) of bisulphite. Reactions were at room temperature for 48 h, unless otherwise stated.

2.1.4. Cysteine, cysteamine and mercaptoethylpyridine

Initial additions used unbuffered cysteamine and cysteine hydrochlorides (0.08 g/g AGE Perloza) and 5 ml water. Subsequently, a 5 molar excess of cysteine±an organic acid was used rather than its hydrochloride. Trichloroacetic, oxalic, formic and acetic acids were used in a 1, 5 or 10 molar excess over cysteine. Cysteamine and mercaptoethylpyridine hydrochlorides (5 molar excess) were mixed with water (1 ml/g) and 1 equivalent of 30% NaOH followed by a 2–5 equivalents of formic or acetic acid. The above solutions were reacted with AGE Perloza samples for 16 h at 60°C.

Cysteamine/formic (5 molar excess) was reacted with 7% AB Perloza for 48–96 h at 60°C, ±ammonium persulphate or benzoyl peroxide (2.5 and 3.6 mg/g Perloza, respectively). The peroxide catalysts were not used subsequently. Cysteamine/formic, cysteamine·HCl and cysteine/formic (10 molar excess) were reacted with 10% AB Perloza for up to 144 h at 70°C. Cysteamine/formic was reacted with 6% AB Perloza for 144 h at 70°C. Control reactions of unmodified Perloza plus cysteamine/formic and allyl Perloza plus ethanolamine (±1 equivalent of formic acid) were for 96 h at 60°C. Cysteine/formic, cysteamine/acetic and mercaptoethylpyridine/acetic (5–10 molar excess) were also reacted with allyl or unmodified matrices by irradiation methods.

2.1.5. Thiolacetic acid and thiophenol

Ethanol or water solvated AGE Perloza was reacted with thiolacetic acid (100 μl) and 5 ml solvent for 24 h at room temperature or 16 h at 60°C [20]. Water solvated 7% AB Perloza (1 g) was used for additions of thiolacetic acid and thiophenol (0.2 ml, mixed with 2 ml water and 3 ml acetone or ethanol) for 90 h at room temperature. After reaction, thiolacetate matrices were washed with 10 volumes each of 0.1 *M* NaOH and water and thiophenol matrices with 10 volumes each of ethanol, 0.1 *M* NaOH and water. Unreacted allyl groups were determined by the MAA back titration used for mercaptoalcohol addition analysis. Thiolacetic acid and thiophenol (0.2 ml each) dissolved in 1–4 ml glacial acetic acid were mixed with 30% AGE Perloza samples (1 g) and reacted by irradiation methods.

2.2. Matrix titrations

A Radiometer ETS822 autotitrator was used to titrate 1 g matrix samples suspended in 5 ml of 1 *M* NaCl. Matrices reacted with mercaptoacids were washed, suction-dried, titrated to pH 8 with Convol 0.1 *M* NaOH and oven-dried by the methods described previously [1]. Bisulphite matrices were treated analogously [1]. GSH matrices were also washed with HCl and titrated with Convol 0.1 *M* NaOH to pH 7. A titration curve of GSH Perloza was obtained by incremental addition of titrant up to pH 11. Cysteamine, amine control and mercaptoethylpyridine matrices were washed and titrated with Convol 0.1 *M* HCl to pH 4 or 3, by the methods described previously [2]. Cysteine Perloza samples were titrated from 11 down to 5 and corrected for a Perloza blank. Titration units are mmol/g (suction-dried) and mmol/g dry (oven-dried).

2.3. Glutathione transferase chromatography

GSH matrices were prepared from 4% AGE Perloza and Sepharose CL6B. Samples were produced by addition of GSH to the allyl matrix, described before. Other matrix samples were treated with aqueous N-bromosuccinimide [2] followed by a 30 min reaction with dilute NaOH, maintained at pH 11.5. GSH was attached to the resulting epoxide intermediate using substitution chemistry [21].

Crude glutathione transferase was prepared by an adaptation of the method of Habig et al. [22]. Frozen bovine liver (260 g) was homogenised for 1 min in a Waring blender with 660 ml water. The homogenate was filtered through glass wool then centrifuged at 13 000 *g* for 45 min. A 100 ml sample of the supernatant was dialysed against 5 m*M* phosphate+100 m*M* NaCl, pH 7 (2×5 l) and the remaining fine particulate matter settled out. The clear supernatant was decanted and passed down a 40 ml column of SP Perloza medium (prepared by the bisulphite addition method described before), equilibrated with 25 m*M* phosphate+100 m*M* NaCl, pH 7 (Buffer A). The peak flow-through of crude glutathione transferase (100 ml) was collected.

Crude enzyme samples (2.5 ml) were applied to 1.8 ml affinity columns (1.5 cm I.D.), equilibrated with Buffer A, and washed at 1 ml/min for 30 min with the same buffer. Elution was with 50 m*M* Tris, pH 8.2+5 m*M* GSH (10 ml) and regeneration with 0.1 *M* NaOH. Elution peak (1–1.5 ml) and whole elution samples were collected. The former were used without modification for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and the latter for activity assay with chlorodinitrobenzene [21] and 280 nm absorbance measurements. Chromatography was monitored with a Biorad Econosystem and LKB recorder. A Pharmacia Phast system and 20% homogeneous gel were used for electrophoresis. Protein bands were developed with the silver stain (development file 210), using 0.033% formalin in the fixing solution. Molecular mass markers were from Sigma.

3. Results and discussion

3.1. Mercaptoacid addition

Reaction of mercaptopropionic acid with 30% AGE Perloza and ammonium persulphate at 60°C resulted in a titration of 0.198 mmol/g, 10% higher than the value obtained after bromination and substitution with sodium sulphite [2]. Removal of the persulphate had little effect and it was not included in subsequent reaction mixtures. It was assumed that light, dissolved oxygen and/or peroxide impurities were sufficient to initiate addition. A much lower titration level was found if a free radical inhibitor, methoxyphenol, was included (Table 1). Replacement of mercaptopropionic acid with mercaptoacetic acid (MAA) resulted in an even higher titration value (Table 1). MAA addition was henceforth the standard method used for allyl group titration.

The use of a 2 or 4 molar excess resulted in 61 and 95%, respectively of the maximum titration. The standard amount of MAA used represented a 5 molar excess or greater over allyl groups up to a 0.28 mmol/g activation level. For very high activation levels, the MAA volume was doubled. MAA addition levels at room temperature were similar to those found at 60°C [20], in contrast to the lower titration

Table 1
Catalyst and inhibitor effects on mercaptoacid addition

Reaction conditions	Titration (mmol/g)	Efficiency (%)
Controls	0.004	n.a.
60°C+Methoxyphenol	0.044	20
Room temperature	0.136	66
60°C	0.186	91
60°C+Ammonium persulphate	0.198	97
MAA, 60°C	0.204	100
N-Ethylmercaptopropionamide	0.004	0
Mercaptoacetate, pH 6	0.004	0

AGE (30%) Perloza was used except for the control samples (Perloza+mercaptopropionic acid or MAA). Mercaptopropionic acid was the ligand used unless otherwise stated. Efficiency was calculated by correction of the titration for the control and comparison to the maximum (MAA) addition level, using values per g Perloza. It was assumed that the molecular mass differences of thiols would not significantly affect wet masses and hence efficiency calculations for this and subsequent tables.

value found with mercaptopropionic acid (Table 1). Reaction of either mercaptoacid with unmodified Perloza produced samples with very low titration values, comparable to those normally found with unmodified Perloza [2]. No addition to 7% AB Perloza occurred if the carboxylic acid was replaced by an amide, N-ethyl-3-mercaptopropionamide, or a carboxylate ion, mercaptoacetate, pH 6. This reactivity in acidic conditions was in contrast to the neutral or alkaline requirements of substitution chemistry [2].

Addition of the other mercaptoacids became less efficient with increasing alkyl chain length, despite reaction at 60°C for 96 h. The addition levels for mercaptobutyric acid and mercaptohexanoic acid were 73% and 14% of MAA, respectively. Similar results were obtained for mercaptobutyric acid using irradiation. No addition of thiosalicylic acid was found after 48 h at 60°C [20].

MAA and mercaptopropionic acid derivatives are simple to prepare and can be used for cation-exchange chromatography or for attachment of affinity ligands by condensation chemistry [23].

3.2. Mercaptoethanol and mercaptopropanediol addition

Addition of mercaptoethanol to AGE Perloza, whether at 60°C or room temperature, was apparently quantitative. Subsequent addition of MAA resulted in titration values lower than unmodified Perloza controls. The sulphur content of one of these samples was 4.45% (1.39 mmol/g dry), also consistent with quantitative addition. Mercaptoethanol addition at room temperature should thus be an effective method for blocking unreacted allyl groups, following ligand attachment. Addition of mercaptopropanediol to 30% AGE Perloza was only slightly less efficient (98%).

3.3. Addition of glutathione and mercaptosuccinic acid

Although significant addition of GSH to an AGE Perloza sample was found by reaction for 16 h at 60°C, the titration value was only 60% of that obtained for MAA [20]. It was assumed that the much greater mass of GSH compared to MAA would not significantly alter wet masses and hence titration/g values. The titration curve of a GSH AGE Perloza sample resembled that of iminodiacetic acid Perloza [2] but with pK_a values approximately 1 unit higher for both titratable groups. An end point of pH 7 was used for subsequent GSH-matrix titrations. More efficient addition (70%) was found with an AGE Perloza of lower activation, but addition of GSH was much less efficient (11%) when the reaction mixture was solvated with 0.1 *M* phosphoric acid rather than water (Table 2). Addition of mercaptosuccinic acid to the same AGE Perloza at 60°C resulted in a similar addition efficiency (71%). The efficiency of both mercaptosuccinic acid and GSH additions was improved by reaction at room temperature for 60 h, using 7% AB Perloza (Table 2).

GSH addition is a simple method for production of affinity matrices [21]. Essentially complete reaction of allyl groups was found at the lower activation levels used for affinity chromatography, despite continuing to use only a 3 molar excess.

Table 2
Efficiency of mercaptosuccinic acid and GSH addition

Matrix/ligand	Titration (mmol/g)	Temperature (°C)	Efficiency (%)
AGE/mercaptosuccinic acid	0.095	60	71
AGE/GSH	0.093	60	70
AGE/GSH/phosphoric acid	0.015	60	11
AB/mercaptosuccinic acid	0.157	20	90
AB/GSH	0.142	20	82

Allyl matrices used were 7% AB and 15% AGE Perloza. Efficiency was determined by comparison with the ''possible'' value obtained by MAA addition (0.174 and 0.134 mmol/g, respectively).

3.4. Bisulphite addition

A high titration value (90% of bromine water value) was found after 30% AGE Perloza (2 g) was mixed for 24 h at room temperature with 0.2 g each of sodium metabisulphite and sodium sulphite [20]. It was presumed that a free radical reaction was involved, although a peroxide catalyst was again not required. Bisulphite was reported to be the reactive species and pH 5.1–6.1 the preferred range for addition to simple alkenes [6]. When sodium metabisulphite was mixed with AGE Perloza at pH 5, the pH dropped during reaction and the addition yield was low (18% of MAA titration). The pH drop was attributed to oxidation of bisulphite to the more acidic bisulphate ion [6]. Greater addition levels were found at higher pH, with a maximum (95–100%) around pH 6 [20]. Equal masses of sulphite and metabisulphite salts produced a suitable reaction pH (6–6.5).

Significantly lower addition levels (46–70% of MAA addition) were found with AB activated Perloza [20]. The reaction volumes were large, because water was used liberally to ensure even mixing. High addition efficiencies (77–100%) were obtained by reaction of AB Perloza (1 g) with 1 ml of 16–20% metabisulphite/16–20% sulphite solution or of the salts with a matrix slurry containing minimal water for mixing (Table 3). Unlike sulphite substitution [2], lower addition levels were found at 60°C, possibly due to a greater rate of bisulphite oxidation compared to addition. At high activation levels, the reaction efficiency of AGE Perloza was still superior (Table 3). Little increase in titration level was found using a bisulphite molar excess greater than 2 for AB or AGE Perloza (Fig. 1).

At high AB activation levels addition of bisulphite was less efficient than MAA but high charge densities were obtained consistently. A probable cause of the lower addition efficiency is charge shielding [2]. Whereas the addition species and matrix product are uncharged for MAA, both bisulphite and the sulphonate matrix product are negatively charged. Therefore electrostatic repulsion combined with steric restrictions could restrict access to some allyl groups, especially at high activation levels. It was presumed that accessibility was improved by the longer spacer arm of AGE, overcoming the steric effects. The level of residual allyl groups after reaction of 7% AB Perloza with bisulphite was too low to demonstrate reliably with bromine water and could not be detected by MAA addition [20]. Because these allyl groups did not react with bisulphite or MAA, they were not expected to interact with macromolecules (and thus affect protein chromatography).

Table 3
Sodium bisulphite addition to allyl Perloza

Chemistry	Titration		Efficiency (%)
	(mmol/g)	(mmol/g dry)	
AB 10%, 60°C	0.070	n.d.	28
AB 10%, 24 h	0.195	1.42	77
AB 10%	0.216	1.59	86
AB 7%	0.179	1.28	99
AGE 30%	0.239	1.43	100
AGE 55%	0.387	1.82	100

A 5 molar excess of metabisulphite, an equal mass of sulphite and the 0.6 ml water was used for AGE Perloza (1.5 g) samples. Pre-formed solutions (1 ml) containing 20% sodium metabisulphite and 20% sodium sulphite were used for reactions with 10% AB Perloza (1 g). Solutions containing 16% of each salt were used for reaction with 7% AB Perloza. Room temperature reactions were for 48 h unless otherwise stated and the 60°C reaction was for 24 h. Efficiency was calculated from wet mass titration data in comparison with MAA addition levels: 0.18, 0.25, 0.238 and 0.387 mmol/g for 7% AB, 10% AB, 30% AGE and 55% AGE, respectively.

The titration curve of bisulphite AB Perloza was typical of a strong cation-exchange matrix, the bed

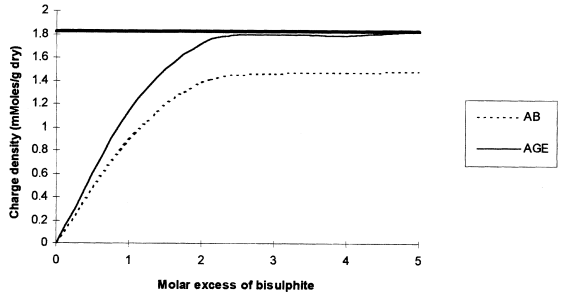

Fig. 1. Effect of bisulphite excess on addition completeness. Matrices used for bisulphite addition were 55% AGE and 14% AB Perloza. The bold line marks the allyl density of 55% AGE Perloza (1.82 mmol/g dry) determined by MAA addition and titration. The allyl density of 14% AB Perloza was slightly higher (1.9 mmol/g dry). Matrices (1.5 g) were mixed with the appropriate molar excess of sodium metabisulphite, an equal mass of sodium sulphite and 0.6 ml water for 48 h at room temperature.

volume stable over a wide range of ionic strength and flow-rates approximately five times greater than those of SP Sepharose Fast Flow at pressures between 0.2 and 2 bar [20]. High protein capacities were obtained, including 39 mg/ml for immunoglobulin G and 89 mg/ml for bovine serum albumin (BSA), and the BSA elution profile was analogous to that of Sepharose Fast Flow [20]. These results showed that allyl activation and bisulphite addition did not compromise the chromatographic properties of Perloza and resulted in normal ion-exchange performance. This preparation of SP cellulose is simpler and safer than that described by Goethals and Natus [24] using propane sultone.

3.5. Nitrogen containing thiols

Although facile reaction was found for GSH, a cysteinyl peptide, addition of cysteine·HCl to an AGE Perloza matrix was limited (10% of MAA titration), after 16 h at 60°C. An even lower addition level was found for cysteamine·HCl (3%). Because addition of GSH was limited in the presence of phosphoric acid, cysteine·HCl was replaced by cysteine ±1 molar equivalent of formic acid. The addition level was five times greater in the presence of formic acid. Formic acid was replaced by acetic acid without a significant change in addition level (43 and 40%) but trichloroacetic and oxalic acids were less effective (30 and 9%). Little difference resulted from use of a 5 or 10 molar excess of formic or acetic acid. Reaction was slow at 60°C and undetectable at room temperature. Similar results were obtained using cysteamine/acetic (39%) and 4-mercaptoethylpyridine/acetic (52%).

Reaction of cysteamine/formic with AB Perloza was not increased by inclusion of ammonium persulphate or benzoyl peroxide. The addition level was increased to 41% by extending the reaction time to 96 h at 60°C. A limit to addition (51%) was found on a highly activated AB Perloza after a 96 h reaction with cysteamine/formic at 70°C and a much lower addition level (11%) was again found with cysteamine·HCl (Table 4). Addition of cysteine/formic at 70°C gave a similar result (46%). Greater cysteamine/formic addition efficiency (79%) was found using an AB matrix of lower activation (0.139 mmol/g). Similar or higher results were obtained by a 3 h irradiation with an infrared lamp (Table 4), although some matrix discoloration was found. Although not an ideal irradiation source, it was presumed this lamp generated sufficient ultraviolet to initiate a radical reaction. The temperature of reaction samples did not exceed 50°C. Efficient addition of cysteine, cysteamine and mercaptoethylpyridine to allyl Perloza, without heating or discoloration, was found using an UV lamp (Table 4). No reaction was found between mercap-

Table 4
Addition levels for cysteamine and cysteine on allyl Perloza

Sample, method	Time (h)	Titration (mmol/g)	Efficiency (%)
Cysteamine, 70°C	24	0.054	22
Cysteamine, 70°C	48	0.098	39
Cysteamine, 70°C	96	0.128	51
Cysteamine, 70°C	144	0.129	52
Cysteine AGE, 70°C	96	0.088	47
Cysteamine AGE, 70°C	96	0.107	57
Cysteamine, IR	3	0.112	60
Cysteamine, UV	4	0.175	94
Cysteine, IR	3	0.138	74
Cysteine, UV	4	0.163	87
Mercaptoethylpyridine, IR	3	0.168	90
Mercaptoethylpyridine, UV	4	0.16	86

Cysteamine and mercaptoethylpyridine (5 molar excess) were dissolved in water+1 equivalent of NaOH and 3 equivalents of acetic acid. Cysteine (10 molar excess) was dissolved in 5 equivalents of formic acid. IR and UV=irradiated with infrared and UV lamps, respectively. The matrix used for heated (70°C) additions was 10% AB Perloza (0.249 mmol/g) except for samples marked AGE (30% AGE Perloza). The same 30% AGE Perloza (0.187 mmol/g) was used for irradiated reactions.

toethylpyridine and unmodified Perloza, using UV catalysis.

Although reaction of formic acid with double bonds has been documented [25], higher temperatures were required. No addition was found between cysteamine/formic and unmodified Perloza or between allyl Perloza and ethanolamine±formic acid after 96 h at 60°C. Free radical addition of an amine to allyl groups was not expected [11] and the ethanolamine results confirmed this. The effect of the organic acids may be catalytic or due to ''masking'' of amine groups.

Although these reactions were less efficient than those of mercaptoethanol, mercaptoacids and GSH, they may be viable. Efficient cysteamine addition has been found at the lower activation levels likely to be used for affinity chromatography. If radiation methods can be scaled up successfully, they would be suitable for industrial as well as laboratory use. The possibility of economic industrial application of free radical addition of thiols to unsaturated compounds has been reported [26]. The specificity of reaction through the thiol group is an advantage over substitution chemistry. Possible uses of mercaptoethylpyridine and cysteamine matrices have been discussed previously [2].

3.6. Thiolacetic acid and thiophenol addition

Thiolacetic acid is reported to be especially reactive with many simple alkenes [8,27]. However, its addition to allyl Perloza was apparently limited (17–22%) compared to MAA. This might be due to proton dissociation in aqueous solutions (the pH of a 1% solution was 1.95). A sample of 30% AGE Perloza (1.42 mmol/g dry) and thiolacetic acid was irradiated for 4 h and hydrolysed with 10 m*M* NaOH. The presence of thiol groups was verified qualitatively with Ellman's reagent [28] but elemental sulphur content was very low (0.06 mmol/g dry).

Thiophenol was reported to be less reactive than MAA with various alkenes but high addition yields were obtained with simple alkenes using ultraviolet irradiation [29]. Some addition of thiophenol to 7% AB Perloza occurred without irradiation, although only 20–25% of the MAA level. A 2.5 h UV lamp irradiation of 30% AGE Perloza (1.42 mmol/g dry) and thiophenol resulted in a higher addition level, 0.88 mmol/g dry (62%) by titration and 0.68 mmol/g dry by elemental analysis. Thiophenol addition could be used to prepare matrices for hydrophobic interaction chromatography.

3.7. Addition of bisulphite and mercaptoethylpyridine to other allyl matrices

Similar results were found with agarose and methacrylate AB matrices (Table 5). Irradiated (infrared lamp) addition of mercaptoethylpyridine/acetic to allyl Fractogel and Sepharose was efficient (≥94%). The titration levels were similar to those obtained by substitution chemistry [2]. The efficiency of its addition to AB Sepabeads was poor and significantly lower than the substitution value [2]. However, preparation of SP ion-exchange derivatives was facile (80–105% of MAA titration values), irrespective of which matrix was chosen. The bisulphite addition limit of AB Perloza was not found with AB Fractogel, despite even higher activation levels. The titration values were corrected for values obtained for unmodified matrices, mixed with bisulphite or MAA. The control titrations were low (<3% of the allyl derivative values) except for Sepabeads (30–40%). A large titration value was also found for Sepabeads prior to mixing with MAA or bisulphite.

3.8. Glutathione transferase chromatography

Glutathione matrices were prepared from AGE Perloza and Sepharose CL 6B by free radical addi-

Table 5
Addition results with other allyl bromide activated matrices

Matrix	MAA	Bisulphite	Mercaptoethylpyridine
Sepharose CL6B	0.14	0.14	0.15
Fractogel	0.41	0.41	0.38
Sepabeads	0.11	0.09	0.04

Activation was with allyl bromide (7%). Allyl matrices (1.2 g) were mixed with 200 μl of MAA at room temperature (48 h). The same conditions were used for addition of a solution (1.5 ml) of sodium metabisulphite and sodium sulphite (20% of each) to 1.5 g allyl matrix. Mixtures of mercaptoethylpyridine/acetic (5 molar excess) and allyl matrix (2 g) were irradiated for 3 h with a Tungsram infrared lamp. Titration values were corrected for blank values obtained for original Sepharose (0.003 mmol/g), Fractogel (0.006) and Sepabeads (0.06 mmol/g).

tion and by bromination and substitution chemistry [2]. Chromatographic profiles using crude glutathione transferase prepared from bovine liver were essentially equivalent for the various matrices and commercial glutathione Sepharose (Fig. 2). The majority of contaminants were not adsorbed under load conditions and glutathione transferase was eluted by 5 m*M* glutathione in 50 m*M* Tris, pH 8.2 [21]. Significant purification was demonstrated by SDS–PAGE of elution peaks (Fig. 3). Total eluted activity varied ±10%, without process optimisation. Some variability may occur due to matrix and ligand density differences. The AGE activation level of

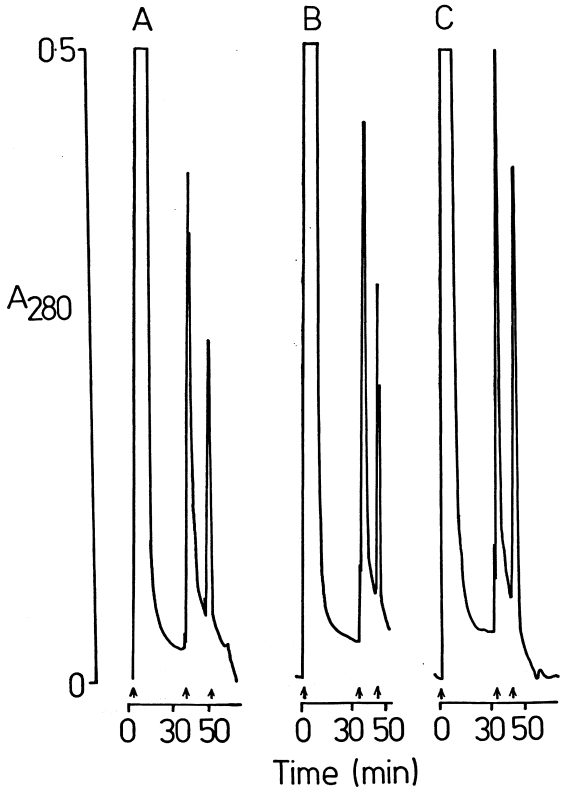

Fig. 2. Glutathione transferase chromatography on various GSH Sepharose matrices. Matrices (1.8 ml) were packed in 1.5 cm I.D. columns and equilibrated with 25 m*M* phosphate+100 m*M* NaCl, pH 7 (Buffer A). Crude glutathione transferase (2.5 ml, prepared as described in Section 2.3) was loaded (first arrow) and the column washed with Buffer A for 30 min. Elution (second arrow) was with 50 m*M* Tris, pH 8.2+5 m*M* GSH (10 ml) and regeneration (third arrow) was with 0.1 *M* NaOH. The traces are for (A) commercial GSH Sepharose CL4B, (B) GSH addition allyl Sepharose CL6B and (C) GSH substitution allyl Sepharose CL6B.

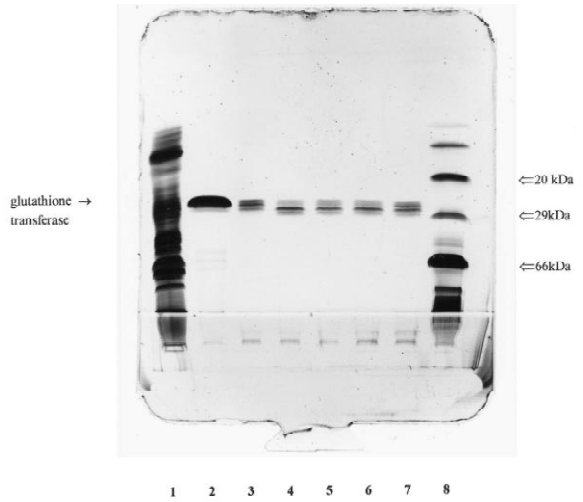

Fig. 3. 20% SDS–PAGE of glutathione transferase samples. Lanes: 1=crude bovine glutathione transferase; 2=equine glutathione transferase; 3=elution from commercial GSH Sepharose 4B; 4=elution from GSH (addition) Perloza; 5=elution from GSH (substitution) Perloza; 6=elution from GSH (addition) Sepharose CL6B; 7=elution from GSH (substitution) Sepharose CL6B; 8= M_r markers: trypsin inhibitor (20 000), carbonic anhydrase (29 000) and BSA (66 000). kDa=kilodaltons.

Perloza was lower than for Sepharose CL6B and consequently GSH densities were lower (16 and 23 μmol/ml, respectively). These results suggested that the products of GSH addition to AGE matrices were useful for purification of crude glutathione transferase. No obvious performance differences were found between matrices produced by addition and substitution chemistry. The AGE activation results in a shorter (7 atom) spacer arm than bisepoxide chemistry (12 atom) of the commercial matrix, which may affect accessibility and capacity.

4. Conclusions

Facile addition of short chain mercaptoacids, mercaptoalcohols, glutathione and bisulphite to allyl matrices has been demonstrated. Medium to high addition efficiency was also found for thiophenol, cysteine, cysteamine and mercaptoethylpyridine, catalysed by heat or irradiation. The resulting matrices can be used for ion-exchange, affinity, hydrophobic and mixed mode chromatography applica-

tions. Allyl activation and free radical addition allows high ligand densities. The chemistry is aqueous compatible and apparently unaffected by hydrolytic side reactions. Because the functional groups which can react are limited compared to nucleophilic substitution, highly specific attachment reactions are possible with multifunctional ligands.

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References

[1] S.C. Burton, D.R.K. Harding, J. Chromatogr. A 775 (1997) 29.
[2] S.C. Burton, D.R.K. Harding, J. Chromatogr. A 775 (1997) 39.
[3] J. Peska, J. Stamberg, J. Hradil, M. Ilavsky, J. Chromatogr. 125 (1976) 455.
[4] J. Stamberg, J. Peska, H. Dautzenburg, B. Philipp, in: T.C.J. Gribnau, R.J.F. Nivard (Eds.), Affinity Chromatography and Related Techniques, Elsevier Scientific, Amsterdam, 1982, p. 131.
[5] J. Stamberg, Sep. Purif. Methods 17 (1988) 155.
[6] P. Gemeiner, M.J. Benes, J. Stamberg, Chem. Papers 43 (1989) 805.
[7] M.S. Kharasch, A.T. Read, F.R. Mayo, Chem. Ind. 57 (1938) 752.
[8] M.S. Kharasch, W.H. Urry, B.M. Kuderna, J. Org. Chem. 14 (1949) 248.
[9] A.R. Stiles, W.E. Vaughan, F.F. Rust, J. Am. Chem. Soc. 80 (1957) 714.
[10] M.S. Kharasch, E.H. May, F.R. Mayo, J. Org. Chem. 3 (1938) 175.
[11] J.I.G. Cadogan, M.J. Perkins, in: S. Patai (Ed.), The Chemistry of Alkenes, Wiley, London, 1964, p. 600.
[12] E.E. Reid, The Organic Chemistry of Bivalent Sulfur Vol. II, Chemical Publishing Co., New York, 1960, pp. 29–35.
[13] C. Walling, Free Radicals in Solution, Wiley, New York, 1957.
[14] G. Sosnovsky, Free Radical Reactions in Preparative Organic Chemistry, MacMillan, New York, 1964.
[15] F.W. Stacey, J.F. Harris Jr., Org. React. 13 (1963) 150.
[16] W.A. Pryor, Mechanisms of Sulfur Reactions, McGraw-Hill, New York, 1962.
[17] J. Brandt, A. Svenson, J. Carlsson, H. Drevin, J. Solid-Phase Biochem. 2 (1977) 105.
[18] M.G. Caron, Y. Srinivasan, J. Pitha, K. Kociolek, R.J. Leifkowitz, J. Biol. Chem. 254 (1979) 2923.
[19] J.P. Danehy, M.Y. Oester, J. Org. Chem. 32 (1967) 1491.
[20] S.C. Burton, Ph.D. Thesis, Massey University, Palmerston North, New Zealand, 1996.
[21] P.C. Simons, D.L. Vander Jagt, Anal. Biochem. 82 (1977) 334.
[22] W.H. Habig, M.J. Pabst, W.B. Jakoby, J. Biol. Chem. 22 (1974) 7130.
[23] P. Cuatrecasas, J. Biol. Chem. 245 (1970) 3059.
[24] E.J. Goethals, G. Natus, Makromol. Chem. 93 (1966) 259.
[25] K. Griesbaum, Angew. Chem., Int. Ed. Engl. 9 (1970) 273.
[26] H.B. Knight, R.E. Koos, D. Swern, J. Am. Chem. Soc. 75 (1953) 6212.
[27] J.I. Cuneen, J. Chem. Soc. (1947) 134.
[28] G.L. Ellman, Arch. Biochem. Biophys. 82 (1959) 82.
[29] J.I. Cuneen, J. Chem. Soc. (1947) 36.