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SEPARATION OF SULPHUR-CONTAINING SUBSTANCES, AMINO ACIDS AND NUCLEOTIDES DURING GEL FILTRATION ON TRISACRYL GF05 BY SPECIFIC GEL INTERACTION

FRIEDRICH KRAUSS and AHLERT SCHMIDT*
Botanisches Institut, Universität München, Menzinger Strasse 67, D-8000 München 19 (G.F.R.)
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SUMMARY

A method has been developed for the separation of sulphur-containing substances of biological interest, amino acids and nucleotides using a specific interaction of these compounds with the filtration gel Trisacryl GF05. Peptides and small molecules with a negative charge showed normal behaviour on this gel. A specific interaction for small molecules containing amino, thiol, and carboxyl groups was observed, depending on the compound used. This specific interaction with the gel is potentially useful for product analysis, for the preparation of sulphur-containing compounds and for the separation of protective thiols from protein extracts.

INTRODUCTION

The study of growth on and the metabolism of sulphur-containing compounds requires an efficient system for the separation and analysis of these compounds. Only a few reports have appeared, which describe a simple method without derivatization for the analysis and preparative separation of cysteine and other thiols[1–3]. We have discovered a specific interaction of a number of small molecules, especially thiol-containing compounds with the gel Trisacryl GF05. This study describes the conditions for the separation of thiol compounds, amino acids and nucleotides using this specific interaction.

MATERIALS AND METHODS

Column conditions

A standard column (75 × 1.4 cm) filled with Trisacryl GF05 was equilibrated with 20 m*M* Tris–HCl (pH 8.0) using a flow-rate of 0.25 ml/min. All substances added were separated using these flow conditions. The eluate was monitored at 254 nm and collected in fractions of 2.5 ml.

If the substances were not eluted by 500 ml of the buffer used, a linear salt-gradient from 0 to 2 *M* sodium chloride in 20 m*M* Tris–HCl (pH 8.0) (200 ml for each reservoir) was used. Afterwards the column was re-equilibrated with the buffer system mentioned above.

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Sample preparation

Either 10 μmol of the nucleotides or 100 μmol of the sulphur-containing substances or amino acids were dissolved in 1 ml of the elution buffer [20 m*M* Tris–HCl (pH 8.0)]. Material which was not soluble in this buffer was dissolved by adding 6 *N* sodium hydroxide and back titrating to a pH of 8.0. This process was necessary for the following compounds: S-carboxymethylcysteine, S-carboxyethylcysteine, S-carbamoylcysteine, lanthionine, djencolic acid, ethionine (pH 9), S-methylcysteine and S-ethylcysteine (both pH 10), and cystine and homocystine (both pH 12).

Sample detection

(1) UV measurement. All nucleotides, mercaptides and thioethers were measured at 260 nm with a Beckmann Model DU 7 spectrophotometer.

(2) Amino acid determinations. Compounds containing amino acid residues were analysed as Ruhemanns purple by normal ninhydrin assay in the following manner[4]: 1 ml of each fraction and 1 ml of the ninhydrin reagent (1.25 g of ninhydrin in 100 ml of water) were heated for 5 min in boiling water. The blue complex formed was measured at 578 nm against a blank containing no amino acid. Cysteine, cystine, cystathionine, S-carabamoylcysteine and S-sulphocysteine formed a yellow complex with this ninhydrin reagent with an absorption maximum at 460 nm. Asparagine was measured at 560 nm, cystamine at 362 nm, djencolic acid at 436 nm, taurine at 375 nm, and β-cyanoalanine at 578 nm. 3-Amino-1-propanesulphonic acid, which did not react with the ninhydrin reagent, was detected by spotting 0.1 ml of each fraction on Whatman No. I paper, spraying with a 0.5% solution of ninhydrin in ethanol and heating for 10 min at 160°C.

(3) Specific determination of cysteine and cysteine derivatives. These sulphur-containing amino acids were detected as their red ninhydrin complexes, with absorption maxima at 560 nm. They were formed under acidic conditions by a modification of the method of Gaitonde[5]. Thus, 20 μl of 0.1 *M* dithioerythritol (DTE), 1 ml of each fraction and 1 ml of Gaitonde reagent (1.25 g of ninhydrin in 20 ml of concentrated hydrochloric acid and 80 ml of acetic acid) were mixed and incubated for 10 min in boiling water. The samples were then cooled rapidly to 0°C (to avoid esterification) and 2 ml of *n*-butanol were added to stabilize the complex. The extinction at 546 nm was measured against a blank containing no cysteine derivative. This test could be used for L-cysteine, D-cysteine, S-sulphocysteine, S-carbamoylcysteine, L-cysteine methyl ester, L-cysteine ethyl ester and N-acetylcysteine (unpublished work and ref. 3).

(4) Thiol determination. All mercaptides were measured by the method of Nashef *et al.*[6] at 460 nm using Ellman reagent (3′,5′-dithiobis-2-nitrobenzoic acid)[7].

(5) Thiosulphate determination. Thiosulphate was determined by the method of Sörbo[8].

(6) Hydrogen sulphide determination. Hydrogen sulphide was determined with methylene blue as described by Siegel[9].

(7) Protein determination. The Biorad protein assay was used with Brilliant Blue G-250 dye according to the method of Bradford[10].

Chemicals

Trisacryl GF05 gel was obtained from LKB (Bromma, Sweden); all sulphur-

TABLE I

SEPARATION OF SULPHUR-CONTAINING COMPOUNDS

Compound	*Detection method**	*Fraction number*
(a) Inorganic compounds		
Hydrogen sulphide	1, 6	27
Sodium [^{35}S]sulphate	×	22
Sodium thiosulphate	5	26
(b) Mercaptides		
2-Mercaptoethanol (ME)	1, 4	45
2-Mercaptoethanesulphonic acid	1, 4	25
Thioglycolic acid	1, 4	26
Thioacetic acid	1, 4	27
1-Thioglycerol	1, 4	43
2,3-Dimercapto-1-propanol (BAL)	1, 4	46
1,4-Dithioerythritol $(DTE)_{red.}$	1, 4	48
Glutathione red. (GSH)	1, 2, 4	25
D-Cysteine	1, 2^1, 3, 4	35
L-Cysteine	1, 2^1, 3, 4	35
N-Acetyl-L-cysteine	2, 4	25
L-Cysteine methyl ester	1, 2, 3, 4	68
L-Cysteine ethyl ester	1, 2, 3, 4	70
DL-Homocysteine	1, 2, 4	42
Cysteamine	1, 2, 4	—/48**
L-Cystine	1, 2^1, 3	43
L-Homocystine	1, 2	40
Cystamine	2^2	—/85**
Glutathione ox. (GSSG)	1, 2	23
(c) Thioethers		
S-Methyl-L-cysteine	2	42
S-Ethyl-L-cysteine	2	41
S-Benzyl-L-cysteine	1, 2	54
S-Carboxymethyl-L-cysteine	2	24
S-Carboxyethyl-L-cysteine	2	24
S-Carbamoyl-L-cysteine	1, 2^1, 3	45
L-Methionine	2	41
L-Methioneamid	2	81
DL-Ethionine	2	42
DL-*meso*-Lanthionine	2	35
DL, DL-*allo*-Cystathionine	2^1	40
L-Djencolic acid	2^3	36
(d) Sulphinic acid		
L-Cysteinesulphinic acid	2	25
(e) Sulphonic acids		
Aminomethanesulphonic acid	2	26
Taurine	2^4	—/41**
3-Amino-1-propanesulphonic acid	2^5	37
L-Cysteic acid	2	26
DL-Homocysteic acid	2	26
S-Sulphocysteine	1, 2^1, 3	24
S-Methyl-L-cysteine sulphoxide	2	27
L-Methionine sulphoximine	2	41
L-Methionine sulphoxide	2	38

* As described under Materials and methods. 2^1 = complex measured at 460 nm; 2^2 = at 362 nm; 2^3 = at 436 nm, 2^4 = at 375 nm, 2^5 = by spraying on paper with 0.5% ninhydrin and heating; × = determined by scintillation counting.

** Fraction numbers of the ninhydrin

containing compounds were purchased from the companies listed by Krauss *et al.*[11]. Nucleotides were purchased from Boehringer (Mannheim, G.F.R.) or Sigma (München, G.F.R.); S-sulphocysteine was obtained from Pierce Eurochemie (Rotterdam, The Netherlands). Other chemicals were purchased from E. Merck (Darmstadt, G.F.R.).

Nomenclature

All chemicals were written according to the nomenclature of the Fluka catalogue (1981/82) obtained from Fluka (Buchs, Switzerland).

RESULTS

Trisacryl GF05 gel has been recommended for gel filtration of small molecules having molecular weights between 200 and 2500 daltons[12]. We noticed, however, that some sulphur-containing compounds chromatographed on this gel in a manner not related to their molecular weight. This study was therefore initiated to analyse in detail this unexpected behaviour of sulphur compounds and amino acids.

Elution properties of sulphur compounds and amino acids

The data for the experiments with sulphur compounds and amino acids are summarized in Tables I and II. Under these conditions dextran blue was eluted in fraction 17 with similar behaviour being found for other high-molecular-weight compounds such as ferritin or bovine serum albumin. Salts were eluted in fraction 22, as demonstrated for sulphate, which was measured as [^{35}S]sulphate. It is evident that most of the sulphur compounds tested were eluted far behind the salt peak, indicating a specific interaction with the gel resulting in a specific retardation for each compound. Typical examples are mercaptoethanol, dithioerythritol or BAL. A specific interaction was also found for thiol-containing amino-acids; however this retardation was influenced also by the length of the carbon chain as shown for cysteine (fraction 36) compared to homocysteine (fraction 42). Blocking of the carboxyl group by meth-

TABLE II

SEPARATION OF L-AMINO ACIDS

Each compound was detected by method 2. Asparagine was measured at 560 nm and β-cyanoalanine at 578 nm, where no visible maxima in the spectra were detectable.

Compound	*Fraction number*	*Compound*	*Fraction number*
Glycine	40	Aspartic acid	24
Alanine	38	Asparagine	38
β-Cyanoalanine	25	Glutamic acid	22
Leucine	41	Glutamine	40
Phenylalanine	47	Lysine	—/32*
Tryptophan	60	Arginine	—/43*
Serine	40	Citrulline	39
Phosphoserine	24	Histidine	46

* Fraction number of the gradient.

ylation resulted in stronger binding, as shown for the cysteine methyl ester (fraction 68), whereas removal of the carboxyl group initiated strong binding, as demonstrated for cysteamine (fraction 48 of the salt gradient). Thioethers of amino acids eluted at fraction 41 (methionine and S-methylcysteine); additional amino groups enhanced binding (methionineamide). This was also true for the non-thiol amino acids lysine and arginine.

Elution properties of nucleotides

The results of some of the experiments with nucleotides are summarized in Table III. Like the separation properties found for amino acids we noticed that nucleotides with no negative charge are eluted late, demonstrating a specific interaction with the Trisacryl GF05 gel. The introduction of negative charges by phosphorylation abolished this interaction more or less completely, with the exception of masked phosphate groups such as adenosine-5′-phosphoramidate, or, in the case of cyclic AMP, made it even more pronounced. Thus nucleosides are eluted late, whereas nucleotides, with the exception of cyclic AMP are eluted early.

pH dependence of the interaction

The influence of the pH on the specific elution pattern was analysed by comparing cysteine, DTE, and S-sulphocysteine at pH 5, 8 and 10 (Fig. 1). It is evident that the elution properties of S-sulphocysteine were not influenced between pH 5 and 8, whereas the interaction of cysteine with the gel was clearly affected by the pH used. This was found also for the chromatographic behaviour of DTE, as shown in

TABLE III

SEPARATION OF NUCLEOTIDES

Detection of each compound at λ = 260 nm.

Substance	*Abbreviation*	*Fraction number*
Adenine		92
Adenosine		70
Inosine		50
Adenosine-3′-monophosphate	3′-AMP	23
Adenosine-5′-monophosphate	5′-AMP	22
2′-Deoxyadenosine-5′-monophosphate	d-5′-AMP	23
Adenosine-3′,5′-monophosphate	cAMP	38
Cytosine-5′-monophosphate	5′-CMP	21
Inosine-5′-monophosphate	5′-IMP	21
Uridine-5′-monophosphate	5′-UMP	21
Uridine-3′,5′-monophosphate	cUMP	23
Guanosine-5′-monophosphate	5′-GMP	22
Guanosine-3′,5′-monophosphate	cGMP	26
Adenosine-5′-monophosphoramidate	APN	27
Adenosine-5′-monophosphosulfate	APS	22
Adenosine-5′-diphosphate	5′-ADP	21
Adenosine-3′,5′-diphosphate	3′,5′-ADP	20
Adenosine-5′-triphosphate	5′-ATP	21
Diadenosine-5′-pyrophosphate	AppA	20

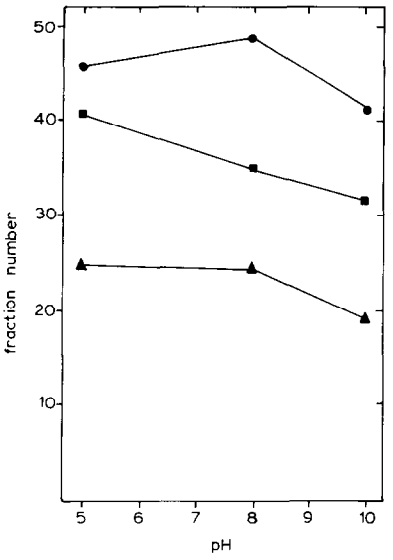

Fig. 1. Influence of pH-value on the separation capacity of the column. The column was eluted either with 20 m*M* sodium acetate–acetic acid (pH 5.0), 20 m*M* Tris-HCl (pH 8.0) or 20 m*M* Tris-NaOH (pH 10.0). Detection was by the methods described in Table I. ■, 100 μmol L-cysteine; ●, 100 μmol DTE; ▲, 100 μmol S-sulphocysteine.

Fig. 1. This demonstrates that the pH is important for the elution of a compound in a specific fraction.

DISCUSSION

The chemical structure of Trisacryl GF05 is characterized by a high degree of hydrophilicity contributed by a secondary amide function and, especially, by the presence of three primary alcohol groups (Fig. 2). These properties explain the fact that the gel separates not only according to molecular weight as suggested by the manufacturers[12], but also as a weak ion exchanger.

The main factors influencing the elution behaviour of the compounds tested seem to be the charge and length of the carbon chain between the terminal charged groups. Compounds with free negative charges, such as $-SO_2^-$, $-SO_3^-$, and $-COO^-$, are eluted very quickly, as there is little or no interaction with the gel matrix. Compounds with net positive charges were bonded stronger (mercaptoethanol).

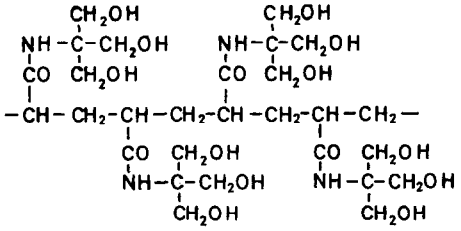

Fig. 2. Structure of Trisacryl GF05.

Under the stated elution conditions the ratio of charges seems to determine the specific elution behaviour. Sulphur-containing, as well as other, amino acids showed some interaction, as could be seen by the elution of fractions 35–45, which could be attributed to a charge balance of the NH_2 and COOH groups (Tables I and II). No significant interaction was detectable for compounds with two negative and only one positive charge, such as S-sulphocysteine, cysteic acid or glutathione. The reverse situation leads to stronger binding, as demonstrated for methionineamide. Masking of the amino groups by acetylation minimized the specific interaction with the gel (N-acetylcysteine) while esterification of the carboxyl group enhanced specific binding (cysteine methyl ester). Removal of the carboxyl group amplified this interaction and thus a salt gradient was needed to elute cysteamine.

The second factor influencing the specific interaction seems to be the length of the carbon chain. This was demonstrated for the two pairs cysteine and homocysteine and aminomethanesulphonic acid and taurine. The increased specific interaction of charged groups with the gel matrix could explain the different elution behaviour for aminomethanesulphonic acid, taurine and 3-amino-1-propanesulphonic acid.

Side chains attached to thioethers did not signicantly affect the elution pattern, as was demonstrated for S-methylcysteine, S-ethylcysteine, methionine and ethionine.

These observations can be extended to nucleotides and related compounds. Strong binding was observed for adenine and adenosine on account of the low net charge resulting from the purine group. Insertion of charged groups such as phosphate decreased the specific interaction, as was seen for instance for 5'-AMP. Cyclic nucleotides such as cGMP and cAMP were specifically retarded on account of their lower negative charges.

Because the charge seems to be of importance for the interaction, the influence of the pH of the elution buffer and the pK_s values of each substance must be considered in order to obtain optimal separation. This was demonstrated for cysteine, dithioerythritol and S-sulphocysteine. High pH values affected the elution pattern on account of the deprotonation of the thiol group and the protonation of the amino group.

The gel, equilibrated as described in this paper, seems to be suitable for different fields of application. It has been used, for instance, in our laboratory to separate cysteine, S-methylcysteine, cysteine methyl ester and S-sulphocysteine from the products of various reactions. Another use for the gel is the separation of cAMP and APN from other adenosine-containing compounds. cAMP and APN are separated very well by this method, even though they have the same net charge and are not separated by electrophoresis[13–15]. The gel may also be used for the separation of certain amino acids and sulphur-containing compounds on a preparative scale, especially if the volatile buffer triethylamine is used[16]. A useful technique seems to be the removal of thiols from protein mixtures, since thiol compounds are specifically retarded by this gel, as has been shown in this paper. Thus, it can be seen that the gel Trisacryl GF05 has several excellent properties, which may be useful for different separation problems.

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