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Note

Adsorption of SH-containing amino acids, peptides and proteins on the 2-nitrophenylhydrazonopropanedinitrile derivative of Spheron Ara

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Reversible covalent chromatography of amino acids, peptides and proteins containing an SH group in the molecule is of interest for both analytical and preparative purposes. Different systems had already been described, *e.g.*, sorption to organomecurial ligands[1], binding via an isothiocyanate group[2] or immobilization on the carriers with activated disulphidic groups[3,4]. The problems of desorption and carrier regeneration have not yet been satisfactorily solved.

Biologically active phenylhydrazonopropanedinitriles (carbonylcyanophenylhydrazones; CCP) react with thiols via the nitrile group; these pH-dependent reactions give addition products that are degraded to the initial substances under alkaline conditions[5,6]. In an earlier paper[7] we described a method for the preparation of phenylhydrazonopropanedinitrile cellulose to which α-toluenethiol and other thiols can be bound. Desorption, however, required drastic conditions (pH 12) incompatible with SH-containing amino acids, peptides and proteins. We therefore prepared a new type of sorbent with 2-nitrophenylhydrazonopropanedinitrile ligand based on the hydroxyalkylmethacrylate gel Spheron.

EXPERIMENTAL

Reagents

Ethanethiol was supplied by Aldrich (Milwaukee, WI, U.S.A.). Oxidized and reduced glutathione (GSSG, GSH), methyl ester of cysteine, N-acetylcysteine and bovine serum albumin (BSA) were purchased from Calbiochem-Behring (Lucerne, Switzerland). L-[^{14}C]Cysteine (activity 27 mCi/mmol) was purchased from Amersham International (Amersham, U.K.). Cysteine, Spheron Ara 1000 (0.040–0.063 mm), SLD-31 scintilation liquid and other chemicals were supplied by Lachema (Brno, Czechoslovakia).

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Synthesis of 4-chlorocarbonyl-2-nitrophenylhydrazonopropanedinitrile (ClNPHPD)

3-Nitro-4-aminobenzoic acid was prepared by nitration of 4-acetylaminobenzoic acid. Diazotation of 3-nitro-4-aminobenzoic acid (concentrated sulphuric acid, sodium nitrite) and subsequent coupling with propanedinitrile (with cooling) gave 4-carboxy-2-nitrophenylhydrazonopropanedinitrile[8,9]. 4-Carboxy-2-nitrophenylhydrazonopropanedinitrile (0.26 g) was refluxed for 1 h with thionyl chloride (4 ml). Unreacted thionyl chloride was removed by vaccuum distillation. Crystallization from dichlororethane gave ClNPHPD (0.32 g, 85% yield, melting point 286–288°C).

Preparation of NPHPD-Spheron

Spheron Ara (10 g) was suspended in anhydrous benzene containing ClNPHPD (1.94 g) and refluxed for 2 h. The product [2-nitrophenylhydrazonopropanedinitrile derivative of Spheron Ara (NPHPD-Spheron)] was washed successively with benzene (90 ml), benzene–acetone (2:1 and 1:2), acetone, acetone–water (2:1 and 1:2) and water.

The amount of ClNPHPD bound was calculated from the difference in the nitrogen contents of NPHPD-Spheron and Spheron Ara. Spheron Ara contained 0.75 mequiv./g of NH_2 groups and NPHPD 0.60 mequiv./g of nitrophenylhydrazonodinitrile, which corresponded to 80% conversion.

Kinetic measurements

Kinetic measurements of the reactions of the reactions of 4-carboxy-2-nitrophenylhydrazonopropanedinitrile (CNPHPD) with ethanethiol, N-acetylcysteine, cysteine methyl ester, cysteine and glutathione were performed spectrophotometrically (Varian, Superscan 3) under conditions of pseudomonomolecular reaction [CNPHPD concentration $2.4 \cdot 10^{-5}$ mol/l, thiols $5 \cdot 10^{-3}$ mol/l, succinate–potassium hydroxide buffers (pH 3.7–7.8), $I = 0.01$ mol/l]. The pK_a value of the CNPHPD derivative was also determined in the above buffers. The pK_a values of SH compounds were taken from the literature[10].

Sorption and desorption of [^{14}C]cysteine

Binding of [^{14}C]Cys to NPHPD-Sephron and its release were studied in citrate buffers at pH 3.4–8.1 (McIlvaine) and at 25°C. Sorption occurred in 3 ml of buffer, 10 mg of sorbent, 0.02 ml of a methanolic solution of [^{14}C]Cys (5 μg/ml) and 0.1 ml of Cys solution (12.5 μg/ml). The release of [^{14}C]Cys from NPHPD-Spheron was determined from 20 mg of sorbent. The time pattern of sorption was followed at pH 5.5; the reactions of [^{14}C]Cys with the sorbent were stopped by 0.1 mol/l HCl. The concentration dependence for [^{14}C]Cys concentrations of 0.2–25.0 μg/ml was measured under the same conditions.

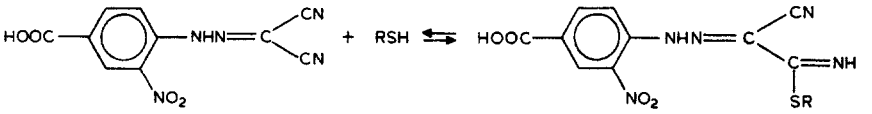
Chromatographic procedures

A column (25 × 1.5 cm I.D.) was packed with preswollen sorbent and it was washed successively with Britton–Robinson buffer of pH 4.0 and 8.0. The last equilibration step was at pH 4.0. Samples of thiols and thiol-containing proteins dissolved in buffer (pH 4.0) were applied to the column. The unbound portion was eluted with buffer (pH 4.0) at a flow-rate of 2.0 ml/min. Substances bound to the carrier were eluted at pH 8.0. Thiol-containing proteins were detected with a UV detector at 280

nm and non-protein thiols were detected with Ellman reagent (DTNB)[11]. Oxidized GSSG was determined by the fluororescence method[12]. Total BSA was determined according to Lowry *et al.*[13].

RESULTS AND DISCUSSION

Kinetic studies of the reactions of CNPHPD with thiols are important in establishing the ability of the prepared sorbent to bind SH-containing amino acids, peptides and proteins reversibly. CNPHPD was chosen for kinetic measurements as ClNPHPD could react with thiols also via the reactive chlorine atom in the molecule:



Reactions were studied under pseudo-monomolecular conditions according to the mechanism described earlier[7]. The phenylhydrazonopropanedinitriles react with SH groups specifically while reactions with amino groups and OH^- ions occur only at high pH values and higher temperatures. The reactions depend on the pK_a values of the imidic proton of the phenylhydrazonopropanedinitrile and the pK_a value of the SH group of the thiol. Kinetic measurements of the reaction of CNPHPD with glutathione showed a pH dependence (Fig. 1), *i.e.*, with increase in pH the final product yield decreased as a consequence of the reaction equilibrium.

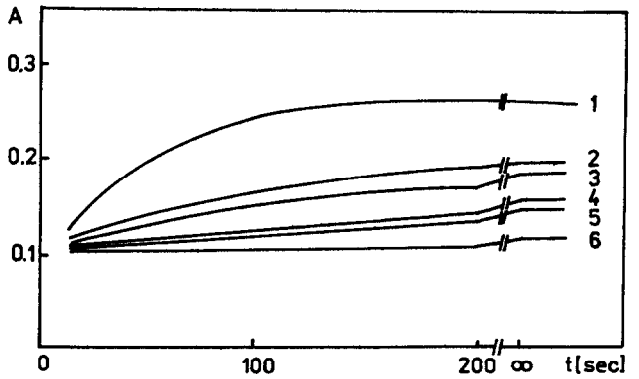


Fig. 1. Reaction of CNPHPD ($2.4 \cdot 10^{-5}$ mol/dm³) with GSH ($5 \cdot 10^{-3}$ mol/dm³) at pH 3.4 (1), 4.3 (2), 5.5 (3), 6.0 (4), 7.2 (5) and 9.0 (6), scanned spectrophotometrically at 420 nm.

Comparison of the first-order rate constants for different SH-containing substances (Table I) indicated that there are pH optima at which the reactions rates are maximal. Except for N-acetylcysteine (which does not occur in biological systems), for CNPHPD a pH of 4.3–5.5 was found to be optimal to give addition products. The second-order rate constants changed in accordance with the pK_a values of the thiols, but a detailed study of such relationships was outside the scape of this work.

TABLE I

FIRST-ORDER RATE CONSTANTS FOR THE REACTION OF CNPHPD WITH THIOLS

Concentration of thiols, $5.0 \cdot 10^{-3}$ mol/dm³; concentration of CNPHPD, $2.4 \cdot 10^{-3}$ mol/dm³.

pH	K_{obs} *(10^{-2} sec^{-1})*				
	Cysteine methyl ester (pK_a 6.5)	*Cysteine (pK_a 8.33)*	*GSH (pK_a 8.6)*	*N-Acetyl-cysteine (pK_a 9.52)*	*Ethanethiol (pK_a 10.61)*
3.4	2.569	0.296	1.045	1.566	0.0324
4.3	2.082	0.898	1.571	0.573	0.0492
5.5	1.564	1.310	2.367	0.564	0.1692
6.0	0.312	0.751	0.756	0.571	0.0420
7.2	0.227	0.477	0.466	0.567	0.0215
9.0	0.124	0.188	0.129	–	–

The study of the effect of pH on the amount of [^{14}C]Cys bound to the polymer showed the highest binding capacity of NPHPD-Spheron at pH 4.0–5.0, which is consistent with the results of kinetic measurements (Fig. 2a). At low pH the time of sorption is short, and at high pH the thiol does not bind to the polymer at all, which was confirmed also by monitoring the release of bound [^{14}C]Cys from the column (Fig. 2b). As the carrier is intended for work with biological materials, the working pH range (4.0–8.0) is advantageous.

The amount of [^{14}C]cysteine bound depends on its concentration in the solution. Determination of the adsorption isotherm showed the maximal adsorption with 0.444 mg of cysteine per milligram of NPHPD-Spheron. Maximal adsorption was obtained under stationary conditions after 30 min (Fig. 3).

After the determination of the above properties of NPHPD-Spheron, we studied sorption in the column using oxidized and reduced glutathione. The results in Table II indicate that GSSG or an excess of GSH is eluted from the column at low pH. At high pH GSH and GSSG are eluted quantitatively from the column.

Fig. 4 shows the separation of bovine serum albumin, which consists of mercaptoalbumin (60–70%) and non-mercaptoalbumin[14]. The first peak eluted at

TABLE II

DEPENDENCE OF THE AMOUNT OF GSH AND GSSG DESORBED FROM THE COLUMN ON THE pH OF THE ELUTION BUFFER

NPHPD-Spheron column, 25 × 1.5 cm I.D.; flow-rate, 1 ml/min.

Sample	*Weight (mg)*	*Amount of desorbed material (mg)*					
		pH of elution					
		4	*5*	*6*	*7*	*8*	*9*
GSH	2	0.95	0.92	1.2	1.7	1.96	1.96
GSSG	1	0.97	0.98	0.98	0.97	0.99	0.96
GSH + GSSG (1:1)	2	0.98	1.01	1.12	1.89	1.89	1.92

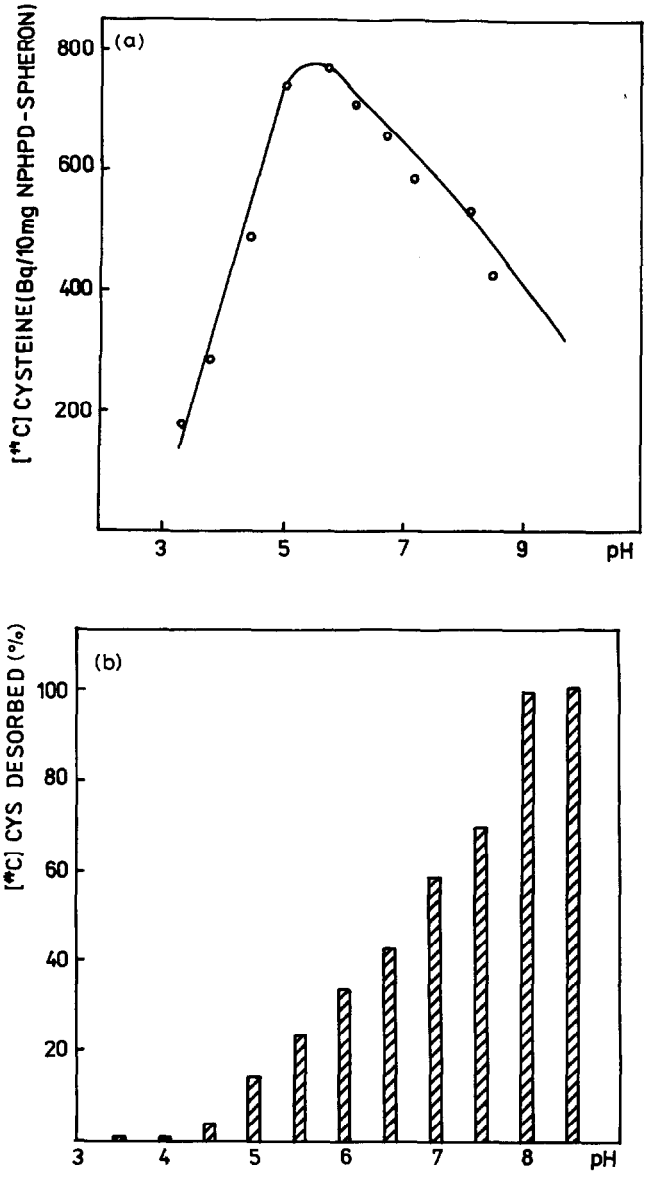


Fig. 2. pH dependence of [^{14}C]Cys sorption on NPHPD-Spheron (a) and its desorption (b).

pH 4.5 was identified as non-mercaptoalbumin and in the second peak, eluted at pH 8, 98% of mercaptoalbumin was determined. In experiments with highly diluted BSA we obtained its concentration in the sample as 2 mg in 5 dm^3.

The properties of the NPHPD-Spheron sorbent can be summarized as follows: (1) the carrier binds reversibly SH-containing amino acids, peptides and proteins via highly reactive ligands of the nitrophenylhydrazonopropanedinitrile type; (2) desorption is quantitative with pH change, which is advantageous compared with other

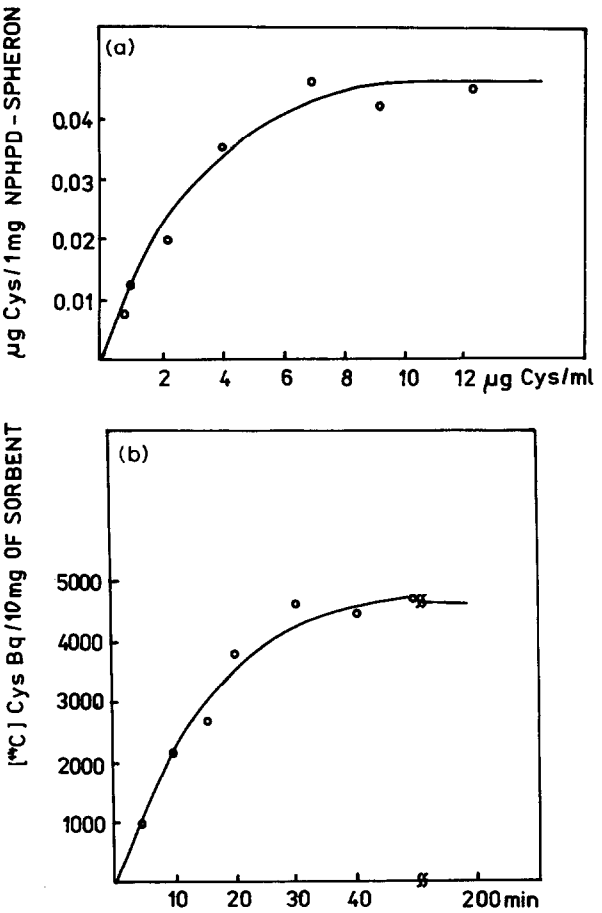


Fig. 3. The time pattern and concentration dependence of [^{14}C]Cys sorption on NPHPD-Spheron. (a) 4 μg/ml of [^{14}C]Cys, pH 5.5; (b) 30 min, pH 5.5 with stirring at 25°C.

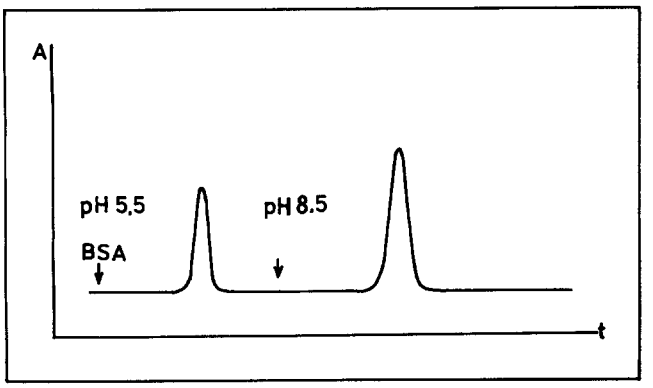


Fig. 4. Separation of bovine serum albumin fractions on a column of NPHPD-Spheron. First peak, mercaptoalbumin: second peak, nonmercaptoalbumin.

methods for the elution of bound substances; (3) the sorbent preserves the outstanding hydrodynamic properties of Spheron Ara. The described method can also be utilized for the preparation of derivatives of cellulose, Sepharose and other matrices.

REFERENCES

1 J. Turková, S. Vavreinová, M. Křiváková and J. Čoupek, *Biochim. Biophys. Acta*, 386 (1975) 503.
2 P. Gemeiner, J. Augustin and Ľ. Drobnica, *Carbohyd. Res.*, 53 (1977) 217.
3 T. A. Egorov, A. Svenson, L. Rydén and J. Carlsson, *Proc. Nat. Acad. Sci. U.S.*, 72 (1975) 3029.
4 K. Brocklehurst, J. Carlsson, M. P. J. Kierstan and E. M. Crook, *Biochem. J.*, 133 (1973) 573.
5 Ľ. Drobnica and E. Šturdík, *Biochim. Biophys. Acta*, 585 (1979) 462.
6 M. Antalík, E. Šturdík, O. Pytela, Ľ. Drobnica and P. Sulo, *Collect. Czech. Chem. Commun.*, 49 (1984) 2807.
7 V. Marko, P. Gemeiner, M. Antalík, E. Šturdík, Ľ. Drobnica and P. Kristian, *React. Polym.*, 1 (1983) 91.
8 J. M. Ross, U.S. Pat., 3 177 247 (1985); *C.A.*, 63 (1965) 545b.
9 H. E. Fierz-David and L. Blangey, *Grundlegene Operationen der Farbchemie*, Springer Verlag, Vienna, 1947, p. 137.
10 J. P. Danehy, K. N. Parameswaran, *J. Chem. Eng. Data*, 13 (1968) 386.
11 G. L. Ellman, *Arch. Biochem. Biophys.*, 74 (1958) 443.
12 P. J. Hissin and R. Hilf, *Anal. Biochem.*, 74 (1976) 214.
13 O. H. Lowry, N. J. Rosenbrough, A. L. Farr and N. J. Ranndall, *J. Biol. Chem.*, 193 (1951) 265.
14 L. O. Andersson, *Biochim. Biophys. Acta*, 117 (1966) 115.