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Note

ISOTHIOCYANATES AS A NEW TYPE OF LIGAND FOR COVALENT CHROMATOGRAPHY OF THIOL PROTEINS[1]

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INTRODUCTION

Immobilization of the reversible inhibitors of the thiol enzymes has been of extensive use in covalent chromatography. Despite this only two types of reactions have so far been utilized: (a) a radical reaction on an organomercuric and (b) an oxidative–reductive one on the disulfidic ligands which were immobilized on a water-insoluble support (1).

The water-insoluble polyisothiocyanates containing free NCS groups are currently the very common supports (2,3). They are used frequently for the covalent immobilization of enzymes, the sequential degradation of peptides, the isolation and purification of antigens and antibodies, and the solid phase radioimmunoassay. In all these cases the biopolymers are covalently bound to polyisothiocyanates through their amino groups by the formation of stable thiocarbamyl compounds.

In contrast to these derivatives (R^1NHCSNHR^2), S-esters of the *N*-monosubstituted dithiocarbamic acids (R^1NHCSSR^2, EDTC), resulting in a reaction of isothiocyanates (R^1NCS, ITC) with thiols (R^2SH), are labile. On the other hand, aliphatic thiols are 1000–50,000 times more

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reactive toward arylisothiocyanates than aliphatic amines or the amino acids (3,4). Because of their lability EDTC are synthetic producers of ITC and consequently powerful inhibitors of the thiol enzymes (3–5). Instability of EDTC is advantageous to the work with ITC as the thiol reagent in the chemistry of proteins (3,6), and for the quantitative release of thiols from cellulose isothiocyanates as well (7).

We now report on the use of cellulose isothiocyanates for covalent chromatography of the thiol enzymes. It has already been mentioned in our preceding paper (7) that only aromatic isothiocyanates covalently bound to a support are interesting for the quantitative release of thiols. For these reasons substituted tolylisothiocyanate bound to cellulose was used in this work. Urease with seven to nine essential thiol groups (8) and papain containing one essential thiol group (9) were used as model enzymes. Both enzymes are selectively inhibited by isothiocyanates (10,11).

MATERIALS AND METHODS

Materials

Urease (urea amidohydrolase, EC 3.5.1.5) from *Canavalia ensiformis* (60 units/mg) and papain (EC 3.4.4.10) from *Carica papaya* (12.5 units/mg) were obtained from Serva Feinbiochemica GmbH, Heidelberg, Germany; powdered cellulose (standard grade) from Whatman Ltd., Maidstone, England; and toluene-2,4-diisocyanate from Merck-Schuchard, Darmstadt, FRG. 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB), *N*-benzoyl-L-arginine-*p*-nitroanilide (BANA), *p*-nitroaniline, and 2-mercaptoethanol (2-ME) were obtained from Fluka AG, Buchs SG, Switzerland. Standard proteins used for the determination of the molecular weight of urease by electrophoresis on polyacrylamide gel (12) were obtained from Calbiochem AG, San Diego, California; Sephadex G-25, G-200, and Blue Dextran 2000 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. The other chemicals and the all-organic solvents were obtained from Lachema, Brno, Czechoslovakia. *O*-(*x*-methyl-4-isothiocyanatophenylcarbamyl) cellulose was prepared and characterized (200 μmol NCS/g) as described previously (7).

Methods

Covalent Chromatography. Urease: Cellulose isothiocyanate (2 g) was equilibrated with 100 mM phosphate buffer (pH 7) containing 1 mM

EDTA. A column was treated with buffer (50 ml) which contained urease in the amount of 1.13 mg/ml. Urease was released from the column by (*a*) 100 mM phosphate buffer (pH 7) containing 1 mM EDTA and 100 mM KCl, and by (*b*) a gradient of 100 mM phosphate buffers (pH 7–9) containing 1 mM EDTA and 10 mM 2-ME. Fractions (5 ml) were collected at a flow rate of 60 ml/h. The appropriate fractions of *a* and *b* containing proteins were combined, dialyzed, and freeze-dried. Cellulose isothiocyanate was regenerated with 10 mM phenyl isothiocyanate in 100 mM phosphate buffer (pH 9) which contained 20% vol/vol of ethanol.

Activated papain. Papain was activated according to Blumberg et al. (13). Enzyme (250 mg/40 ml) was desalted on Sephadex G-25 using nitrogenated buffer (pH 4.3), containing 25 mM EDTA as eluent. A solution of papain (pH 5) was applied to a column of cellulose isothiocyanate (5 g) equilibrated with 100 mM acetate buffer (pH 5) containing 2 mM EDTA. Elution of the column with (*c*) 100 mM acetate buffer (pH 4.3) containing 2 mM EDTA and 300 mM KCl, and with (*d*) 100 mM acetate buffer (pH 5) containing 30 mM 2-ME and 2 mM EDTA removed papain which was collected in 5-ml fractions at a flow rate of 30–40 ml/h. The combined fractions were desalted by gel chromatography on Sephadex G-25 and worked up as described previously. The regeneration of cellulose isothiocyanate was performed in the same way.

Enzymatic Activity, Concentration of Proteins and Their Thiol Groups. The urease activity was determined spectrophotometrically at 436 nm (25°) after nesslerization using the procedure of Carlsson et al. (14). The amidolytic activity of papain toward BANA was determined at 405 nm (37°C) according to Kimmel and Smith (15). The concentration of proteins was established from absorbances at 278 nm (urease $A^{1\%}_{1\,cm} = 0.75$) (8) and at 280 nm (papain $A^{1\%}_{1\,cm} = 25$) (9) or by Lowry's micromethod (16). The concentrations were calculated using molecular weight 480,000 for urease (8) and 23,000 for papain (9). Concentration of the thiol groups was determined by spectrophotometric titration with DTNB (17) using a value $\varepsilon_{412} = 13{,}600\ M^{-1}\ cm^{-1}$.

SDS Gel Electrophoresis. Electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS) and dithiothreitol together with a method for determination of molecular weights has been described by Russ and Poláková (12). The current density was 2–5 mA on one gel at 30–40 V for 10–20 h at 25°C with 7.5% wt vol of polyacrylamide gel. A sample (10–20 μg) was applied.

Gel Chromatography. Gel chromatography of urease on Sephadex G-200 was performed according to Fishbein and Nagarajan (18).

RESULTS

Using cellulose tolylisothiocyanate (7) two main fractions (Fig. 1) were obtained from commercial urease. The fractions differed considerably in the specific activities, in concentration of the thiol groups, and in the number of zones on SDS gel electrophoresis. The fraction eluted with buffer *a* was almost inactive and also had a lower concentration of thiol groups than the parent enzyme (6 mol SH/mol protein). The fraction eluted with buffer *b*, however, contained an enzyme that had 30 times higher activity; the concentration of thiol groups was 30.1 mol SH/mol protein. The specific activity of the isolated enzyme was approximately two times higher than that of the urease which was obtained after rechromatography on Sephadex G-200 (18). Gel electrophoresis of the parent urease showed two spots corresponding to the molecular weights of the dissociated hexamer. The purified enzyme gave only one intensive spot corresponding to a monomer (mol. wt. 80,000).

An immobilized inhibitor (arylisothiocyanate) also bound papain selectively which was activated according to Blumberg et al. (13) (45 units/mg, 0.35 mol SH/mol protein). Binding was carried out under

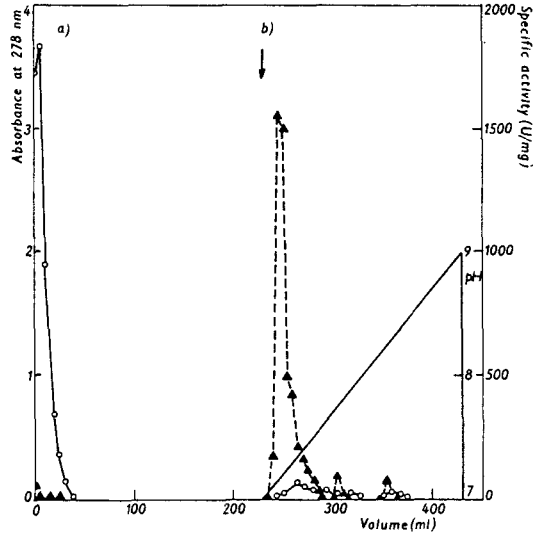

FIG. 1. Covalent chromatography of urease on cellulose isothiocyanate. Absorbance at 278 nm (○—○); specific activity, μmol of NH_3/mg (▲- - -▲). Buffers *a* and *b* were used for elution.

slightly acidic conditions. A portion that was not bound did not show any activity (~3 units/mg, 0.02 mol SH/mol protein) and came through in the eluate. Elution of the column with buffer (pH 5) that contained thiol released the enzyme with an activity of 115 units/mg toward the BANA substrate. This fraction contained 0.95 mol SH/mol protein.

DISCUSSION

On cellulose arylisothiocyanates the covalent chromatography of thiol proteins proceeds, as in the preceding experiments (4,6,7), through the formation and decomposition of EDTC. The appropriate models of EDTC [R^1 = phenyl, 4-bromophenyl, *O*-(*x*-methylphenylcarbamyl) cellulose; R^2 = *n*-propyl, 2-hydroxyethyl, benzyl, glutathionyl, D-glyceraldehyde-3-phosphate dehydrogenase] were characterized by the dissociation constant $pK_a = 8.9–9.2$, and by the rate constants of formation, $k_{-1b} = 600–1100\ M^{-1}\ s^{-1}$, and decomposition, $k_{+1b} = 0.002–0.01\ s^{-1}$. In this case the S-ester part (R^2) was formed by the cysteine residues and *O*-(*x*-methylphenylcarbamyl) cellulose was the substituent on nitrogen (R^1). If only EDTC is formed, a thiol residue can be removed quantitatively (4,7). In the experiments with aminothiols (thiol enzymes are considered for them too) it is not possible to exclude completely any formation of the stable thiocarbamyl compound where R^2 can be a residual *N*-terminal amino acid or lysine. This might explain the 25–35% loss of the binding capacity of *O*-(*x*-methyl-4-isothiocyanatophenylcarbamyl) cellulose after the use and regeneration by phenylisothiocyanate.

Urease obtained by covalent chromatography on the cellulose tolylisothiocyanate was not homogeneous with respect to the specific activities though a protein macromolecule was homogeneous. Highly purified urease is known for the high content of thiol groups (33–37) which may react with the thiol reagents. The polymer contains, on the average, seven to nine essential thiol groups (8). Differences in the specific activities may arise from the number of thiols or essential thiol groups which are present in a particular urease molecule. Gel electrophoresis of urease in a very unusual way, namely in the presence of SDS and dithiothreitol, was carried out because of the possible presence of 12 polymeric and dissociated forms in the investigated enzyme.

Mercaptopapain could be prepared by covalent chromatography as described by Blumberg et al. (13) only after activation. Covalent chromatography of the thiol enzymes on cellulose arylisothiocyanates is limited by the proper selection of the pH at which the enzyme is bound on the support. This is necessary for two reasons: (i) to ensure reaction only with

the thiol groups of enzyme; and (ii) to perform binding on the support at the pH at which the enzyme retains its full activity. The latter condition can be overlooked by the isolation and purification of thiol peptides. This was confirmed in the experiments with the hydrolysates of active papain which will be continued in the future.

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