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PURIFICATION OF THIOREDOXIN FROM *ESCHERICHIA COLI* AND BACTERIOPHAGE T4 BY IMMUNOADSORBENT AFFINITY CHROMATOGRAPHY

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SUMMARY

Specific immunoadsorbents for thioredoxin from *Escherichia coli* and phage T4 were prepared by coupling the γ-globulin fractions of rabbit antithioredoxin antisera to Sepharose. The immunoadsorbents could be used for rapid and efficient purification of thioredoxin from crude bacterial extracts. The T4 thioredoxin immunoadsorbent did not bind *E. coli* thioredoxin and *vice versa*. This permitted the isolation of both *E. coli* and T4 thioredoxin from phage-infected cells by two consecutive chromatography steps on the specific immunoadsorbents.

INTRODUCTION

Affinity chromatography of proteins utilizes an insoluble compound or macromolecule with selective affinity for a given protein. An immunological application of this method involves the binding of an enzyme to specific antibodies, which have been covalently coupled to Sepharose[1].

We recently obtained potent rabbit antisera specific for thioredoxin from *Escherichia coli* or the thioredoxin induced in *E. coli* upon phage T4-infection[2]. The specific antibodies, which did not cross-react with their heterologous thioredoxin[2], were used to develop a simple and rapid immunoadsorbent preparation method for these thioredoxins. The method permits the parallel isolation of *E. coli* and T4 thioredoxin from a crude extract of phage-infected cells by two successive affinity chromatography steps.

MATERIALS AND METHODS

E. coli B3 cells and phage T4 *am* 122-infected *E. coli* B cells were obtained by the previously described methods[3,4]. Highly purified thioredoxin reductase from *E. coli* B after Sephadex G-100 chromatography was a kind gift from Dr Lars Thelander, Stockholm, Sweden. Diaflo ultrafiltration membranes were bought from Amicon Corp.

NADPH and 5,5′-dithiobis-(2-nitrobenzoic acid) were products of Sigma Chemical Co. Cyanogen bromide was from Eastman Organic Chemicals. Sepharose 4B was a product of Pharmacia. Antiserum against *E. coli* and T4 thioredoxin were prepared by immunization of rabbits as described previously[2].

Protein determination

Protein was determined by reading the absorbance at 280 and 310 nm in a Zeiss PMQII spectrophotometer. An $A_{280\ nm}$ unit is defined as the amount of protein in 1.00 ml of solution which will give a difference in value of 1.00 between the absorbances at 280 and 310 nm.

Concentration of column eluates

This was accomplished by ultrafiltration using Diaflo membranes, Type UM 20E.

Assays for thioredoxin

During the preparation *E. coli* and T4 thioredoxins were measured by an enzymatic method in which the reduction of thioredoxin by NADPH in the presence of thioredoxin reductase is coupled to the rapid reoxidation of thioredoxin by 5,5′-dithiobis-(2-nitrobenzoic acid) (Method 2 in refs 4 and 6). Although both *E. coli* and T4 thioredoxins are substrates for the bacterial thioredoxin reductase, their enzymatic activities could be determined separately by the inclusion of 5 μl T4 or *E. coli* thioredoxin antiserum in the assay mixture[2]. Aliquots of crude bacterial extract were heated for 2 min in a boiling water bath before assay[7]. This treatment removed interfering NADPH oxidase activity, but the recovery of thioredoxin was better than 80%.

The purity of the *E. coli* or T4 thioredoxin finally obtained was determined as the amount of NADPH oxidized per $A_{280\ nm}$ unit, in the presence of thioredoxin reductase (Method 3 in refs 4 and 6).

Preparation of immunoadsorbents

All operations were carried out at +4 °C. The γ-globulin fraction of an antiserum against *E. coli* or T4 thioredoxin[2] was isolated by repeated precipitation with $(NH_4)_2SO_4$ to 40% satn of the salt and then extensively dialyzed against 0.2 M sodium citrate (pH 6.5) and brought back to the original serum volume. Sepharose was activated with cyanogen bromide (300 mg/ml packed Sepharose) by the procedure of Cuatrecasas[8]. To 100 ml of the activated Sepharose, 40 ml of the γ-globulin fraction (about 780 $A_{280\ nm}$ units) were added and the mixture was slowly stirred for 24 h as a 50% slurry in 0.2 M sodium citrate (pH 6.5). One vol. of 0.1 M 2-aminoethanol (pH 7.0) was then passed through the column to ensure complete loss of reactive agarose groups. The immunoadsorbent was submitted to one cycle of the adsorption–desorption procedure before use.

RESULTS AND DISCUSSION

The thioredoxin immunoadsorbents were prepared by coupling the γ-globulin fraction to the Sepharose at pH 6.5 although this is not the optimal pH for the coupling of proteins[8]. The pH value was chosen in order to preserve as much as pos-

sible of the biological activity of the antibodies, which still retained 70% of their original capacity to neutralize the enzymatic activity of thioredoxin[2]. Since the thioredoxin antisera contained very high titers of antibodies, the γ-globulin fraction obtained from one or two rabbits would be sufficient to give a column with a capacity for binding 1 μmole of thioredoxin.

Purification of E. coli thioredoxin

E. coli thioredoxin can be efficiently isolated from a crude bacterial extract[3] by the specific immunoadsorbent, as shown in Fig. 1. Adsorption was performed in 0.05 M Tris–HCl buffer (pH 8.0), after which the column was extensively washed with 1 M Tris–HCl buffer (pH 8.0). *E. coli* thioredoxin was then desorbed in 0.1 M acetic acid (adjusted to pH 2.2 by the addition of formic acid), and the acidic fractions were immediately neutralized with 1 M $NaHCO_3$. This acidic elution procedure used to dissociate the antigen–antibody complexes involves a reversible denaturation of the protein antigen which thioredoxin can withstand. At this stage thioredoxin could be obtained with a purity of 80% without loss of enzymatic activity as assayed by the thioredoxin reductase method. Chromatography on Sephadex G-50 (ref. 9) removed remaining impurities to give homogeneous thioredoxin, which could be crystallized[10]. The method offers a simple purification procedure as compared to the multistep procedure involving heat treatment and three column chromatography steps[6] normally

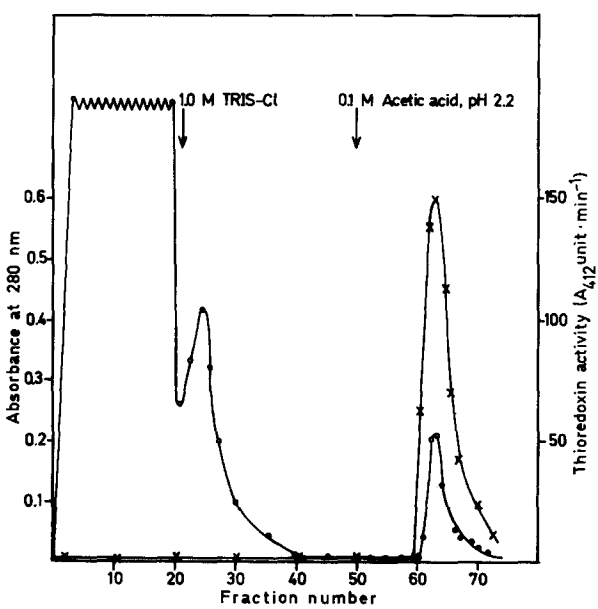

Fig. 1. Isolation of *E. coli* thioredoxin on an immunoadsorbent column. The column contained the γ-globulin fraction from 40 ml of rabbit antiserum. To the column, 5100 $A_{280\ nm}$ units (430 ml) of an acetic acid supernatant obtained from *E. coli* B3 (ref. 3) were added. The column was washed with 100 ml 0.05 M Tris–HCl buffer (pH 8.0) followed by 500 ml 1.0 M Tris–HCl buffer (pH 8.0) and 100 ml 0.05 M Tris–HCl buffer (pH 8.0). The adsorbed protein was eluted with 200 ml of 0.1 M acetic acid adjusted to pH 2.2 by formic acid. Fractions of 20 ml were immediately neutralized by the addition of 1.0 M $NaHCO_3$. ○—○, $A_{280\ nm}$; ×—×, thioredoxin activity in 50-μl aliquots of the fractions.

used in the purification of *E. coli* thioredoxin. The immunoadsorbent has retained 70% of its original capacity after 30 cycles of adsorption–desorption over a period of 10 months.

Purification of T4 and E. coli thioredoxin from phage-infected cells

The purification was started from 140 g of *E. coli* cells infected with phage T4. A crude preparation of T4 and *E. coli* thioredoxin was obtained in the $(NH_4)_2SO_4$ precipitable fraction between 35 and 85% satn of the salt, as described earlier for T4 thioredoxin[4]. The thioredoxin-containing fraction was equilibrated with 0.05 M Tris–HCl–0.001 M EDTA buffer (pH 8.0) on a column of Sephadex G-25 and chromatographed on the T4 thioredoxin immunoadsorbent column. T4 thioredoxin was completely adsorbed on the column while *E. coli* thioredoxin was recovered in the peak of unadsorbed proteins (Fig. 2). After thorough washing of the column the T4 thioredoxin could be desorbed by acid dissociation; the acidic fractions were immediately neutralized by $NaHCO_3$. At this stage T4 thioredoxin was 70% pure and could be obtained in a homogeneous form by an additional chromatographic run on Sephadex G-50 (Table I and ref. 4) or CM-cellulose[11].

The *E. coli* thioredoxin, contained in the unadsorbed protein fraction of the T4 thioredoxin immunoadsorbent column, was now adsorbed to the *E. coli* thiore-

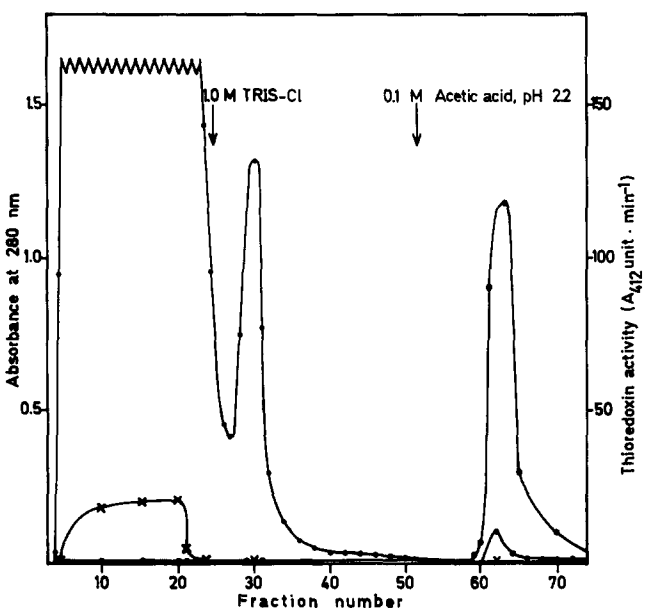

Fig. 2. Separation of T4 and *E. coli* thioredoxin on a T4 thioredoxin immunoadsorbent column. The column (4 cm × 8 cm) contained 1000 $A_{280\ nm}$ units of rabbit anti-T4 thioredoxin γ-globulin fraction. To the column, 18 000 $A_{280\ nm}$ units of a crude preparation of T4 and *E. coli* thioredoxin were added in a volume of 400 ml. The column was washed with 200 ml 0.05 M Tris–HCl buffer (pH 8.0) followed by 500 ml 1.0 M Tris–HCl buffer (pH 8.0) and 100 ml 0.05 M Tris–HCl buffer (pH 8.0). Adsorbed protein was eluted with 200 ml 0.1 M acetic acid, adjusted to pH 2.2 by formic acid; the acidic fractions were immediately neutralized by the addition of half a volume of 1.0 M $NaHCO_3$. ●—●, $A_{280\ nm}$; ○—○, T4 thioredoxin activity; ×—×, *E. coli* thioredoxin activity. Both activities are expressed as activity in 20-μl aliquots.

TABLE I

PURIFICATION OF T4 AND *E. coli* THIOREDOXIN FROM PHAGE-INFECTED CELLS

The data refer to 140 g of *E. coli* B cells infected with phage T4 *am* 122. The crude extract contained 15 000 $A_{280\ nm}$ units obtained in the $(NH_4)_2SO_4$ fraction between 35 and 85% satn. Activities were determined by measuring the amount of NADPH oxidized in the presence of thioredoxin reductase (Method 3 in refs 4 and 6).

Fraction	*Protein*	*T4 thioredoxin*		*E. coli thioredoxin*	
		Total activity	*Spec. act.*	*Total activity*	*Spec. act.*
	($A_{280\ nm}$ units)	*(μmoles)*	*(nmoles/$A_{280\ nm}$ unit)*	*(μmoles)*	*(nmoles/$A_{280\ nm}$ unit)*
T4 thioredoxin immunoadsorbent	5.8	0.64	111	—	—
Sephadex G-50	3.4	0.54	158	—	—
E. coli thioredoxin immunoadsorbent	7.8	—	—	0.42	54
Sephadex G-50	4.7	—	—	0.34	72

doxin immunoadsorbent column in the same manner as described above (see also Fig. 1). The acid-dissociable bacterial thioredoxin obtained in this step was 75% pure and could be purified to homogeneity by an additional chromatographic run on Sephadex G-50 as described above (Table I and ref. 9).

The parallel preparation of *E. coli* and T4 thioredoxin by two consecutive immunoadsorbent chromatography steps permits rapid and reproducible isolation of active thioredoxins, which in this single step can be purified about 200 times. The immunoadsorbent columns should also permit the isolation of mutant thioredoxins of *E. coli* and phage T4, which are enzymatically inactive but still retain immunological activity.

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