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NOTE

Overexpression of *Escherichia coli* Thiol Peroxidase in the Periplasmic Space

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Abstract Overproduction of *Escherichia coli* thiol peroxidase in the periplasmic space was achieved by locating the appropriate gene on a downstream region of the strong T7 promoter. *E. coli* strain BL21 carrying the recombinant plasmid pSK-TPX was induced by IPTG, lysed, and analyzed by SDS-polyacrylamide gel electrophoresis. A large amount of the overexpressed thiol peroxidase was located in the periplasmic space. A homogeneous thiol peroxidase was obtained from *E. coli* osmotic shock fluid by simple one-step gel permeation chromatography.

Key words: Thiol peroxidase, overexpression, periplasm, T7 promoter, *E. coli* BL21, induction, gel permeation chromatography

Exposure of bacteria to endogenous and exogenous reactive oxygen species, such as peroxides, superoxide anions, and hydroxyl radicals from various oxidation reactions may have harmful effects on cellular constituents due to oxidation of lipids, proteins, and nucleic acids [4-6]. To prevent oxidative damage due to reactive oxygen species, cells are equipped with a number of antioxidant enzymes, including catalase, peroxidase, and superoxide dismutase [7].

The existence of the novel antioxidant enzyme p20 in the *E. coli* periplasmic space has been reported. P20 showed peroxidase activity linked with an *in vivo* thiol-regenerating system, a thioredoxin-thioredoxin reduction system [2, 3]. The gene encoding p20 was also isolated from an *E. coli* K12 genomic DNA library and the complete nucleotide sequence was determined [2, 10]. Since *E. coli* has a number of proteins showing peroxidase activity [2, 9], it is tedious and difficult to purify the 20-kDa antioxidant protein from an *E. coli* crude extract.

In this study we overexpressed *E. coli* thiol peroxidase in the periplasmic space using a bacteriophage T7 RNA polymerase/promoter system [14] for convenient protein purification. Overproduction of biologically active thiol peroxidase in the periplasmic space and a simple purification method from *E. coli* osmotic shock fluid are described.

E. coli BL21 [14] and the pBluescript II SK plasmid (Stratagene, La Jolla, CA, U.S.A.) were used for expression of the *E. coli* thiol peroxidase gene. *E. coli* cells were grown in Luria-Bertani (LB) medium for transformation, or in NZCYM broth for gene expression. Ampicillin was added at 200 μg/ml for plasmid maintenance. The pTX plasmid containing a 2.2-kb *Bgl*II DNA fragment [10] was used as a PCR template for amplification of the thiol peroxidase gene by using a forward primer (5'-GGGAATTCTAGCGTTTGCTGTG-TTATT) and a reverse primer (5'-GGGCTGCAGGCA-TATTAAATTATGC). The PCR reaction was performed using *Pfu* DNA polymerase. The amplified DNA fragment was digested with *Eco*RI and *Pst*I restriction enzymes, then ligated with pBluescript II SK plasmids treated with the same restriction enzymes. The standard procedures of Sambrook *et al.* [13] were used for construction of the recombinant plasmid pSK-TPX. *E. coli* BL21 strains carrying the pSK-TPX and pBluescript II SK plasmids were grown to mid-log phase in NZCYM broth containing ampicillin. Parallel strains were additionally cultured after additions of 1 mM isopropyl-β-D-thiogalactoside (IPTG).

Proteins were separated by electrophoresis in 12% (w/v) SDS-polyacrylamide gel (SDS-PAGE) by the method of Laemmli [12]. For the analysis of proteins in whole-cell extracts, 1 ml of cell culture was suspended in 200 μl of a 1× gel loading buffer (50 mM Tris-HCl,

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pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), boiled for 5 min, and centrifuged for 5 min in an Eppendorf microcentrifuge. A total of 10 µl of the solubilized material, equivalent to 50 µl of the cell culture, was loaded per lane. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250. Periplasmic proteins were released by the chloroform shock procedure [1]. To release the periplasmic proteins, 1 ml of IPTG-induced *E. coli* BL21 culture was harvested by Eppendorf centrifuge, resuspended by brief vortexing in the residual medium, then 20 µl of $CHCl_3$ was added. After standing for 15 min at room temperature, 100 µl of 10 mM Tris-HCl (pH 8.0) was added, then the cells were separated by centrifugation. The supernatant and the residual cell pellet were used for analysis of periplasmic and cytoplasmic proteins, respectively. For large scale protein purification the cold osmotic shock method was used as described previously [8]. Ammonium sulfate was added to precipitate the proteins in the cold osmotic shock fluid to a final saturation of 80% at 0°C. The precipitate was dissolved in 2 ml of 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM KCl and applied to a Sephacryl S-200 HR column equilibrated with same buffer. The thiol-dependent antioxidant activity of *E. coli* thiol peroxidase was determined by monitoring their activity to inhibit the inactivation of *E. coli* glutamine synthetase [11].

Purification of a 20-kDa novel antioxidant protein showing thioredoxin-linked thiol peroxidase activity and cloning of the gene from *E. coli* has been reported [2]. For development of a convenient protein purification method from *E. coli*, thiol peroxidase was overproduced using a T7 RNA polymerase/promoter system. To construct a plasmid for overproduction of thiol peroxidase the pTX plasmid was used as a template for amplification of the thiol peroxidase structural gene retaining its own promoter with *Pfu* DNA polymerase. We generated *Eco*RI and *Pst*I restriction sites at the 5' and 3' ends of the thiol peroxidase gene so that the PCR product could be inserted within the multicloning site of the pBluescript II SK plasmid thereby creating plasmid pSK-TPX. By this procedure the thiol peroxidase gene was placed downstream of the T7 promoter of the pBluescript II SK vector. The constructed recombinant plasmid DNA was transformed into *E. coli* BL21 carrying the T7 RNA polymerase gene (λDE3 lysogen) controlled by the *lac*UV5 promoter.

Cultures of *E. coli* BL21 carrying the pSK-TPX and pBluescript II SK plasmids were grown to mid-log phase at 37°C, and 1 mM IPTG was added to induce the thiol peroxidase protein. The cells were induced up to 3 h, harvested, lysed, and analyzed by 12% (w/v) SDS-PAGE. As illustrated in Fig. 1A, an increased band in

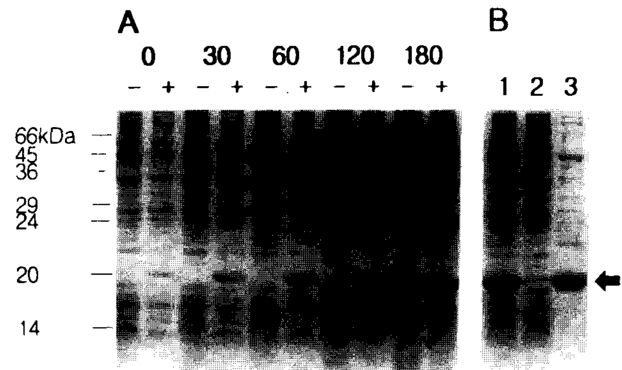

Fig. 1. (A) Time course of induction for *E. coli* thiol peroxidase and (B) location of thiol peroxidase overproduced in *E. coli* BL21 (pSK-TPX).

(A) *E. coli* BL21 containing the pBluescript II SK and pSK-TPX plasmids was grown at 37°C and induced by addition of 1 mM IPTG. Samples were taken from cultures at 0, 30, 60, 120, and 180 min after induction. - Lanes, *E. coli* BL21 (pBluescript II SK); + lanes, *E. coli* BL21 (pSK-TPX). The time after induction corresponding to each sample is indicated at the top of the gel. (B) Chloroform shock was applied to 1 ml of bacterial cells after 2-h IPTG induction. Lane 1, total cell extracts equivalent to 50 µl of bacterial culture; lane 2, residual cell extracts equivalent to 50 µl of culture after chloroform shock treatment; lane 3, chloroform shock fluid equivalent to 50 µl of culture. The position of the overproduced protein is indicated by the arrow.

intensity corresponding to the p20 protein appeared in cell extracts of induced *E. coli* BL21 containing the pSK-TPX plasmid on SDS-PAGE analysis. The new protein was induced to a maximum level in 2 h, based on SDS-PAGE analysis of total cell extracts (Fig. 1A). Thiol peroxidase constituted over 15% of the total cellular proteins in 2-h IPTG-induced *E. coli* cultures, as determined by densitometric scanning of the Coomassie Blue-stained polyacrylamide gel (data not shown).

In order to determine whether thiol peroxidase is produced as an insoluble form in *E. coli*, a whole cell extract of induced cells that had been disrupted by deoxycholate and brief sonication was pelleted by centrifugation. The precipitate was solubilized in either a 2 M or 8 M urea solution. A protein band corresponding to 20 kDa was detected only in supernatant by SDS-PAGE analysis, suggesting that the protein overproduced in *E. coli* BL21 did not form an inclusion body in the cytoplasm (data not shown). The thiol peroxidase protein in wild type *E. coli* K12 was reported to be present in the periplasmic space where it could have a principal antioxidative role in removing exogenous peroxides [2]. To determine the location of overexpressed thiol peroxidase in *E. coli* BL21, an induced *E. coli* culture was subjected to chloroform shock in order to release the periplasmic proteins [1]. Thiol peroxidase appeared as a major band in the periplasm, as previously reported in wild type *E. coli* K12 (Fig. 1B, lane 3), which made our protein purification procedure considerably simpler.

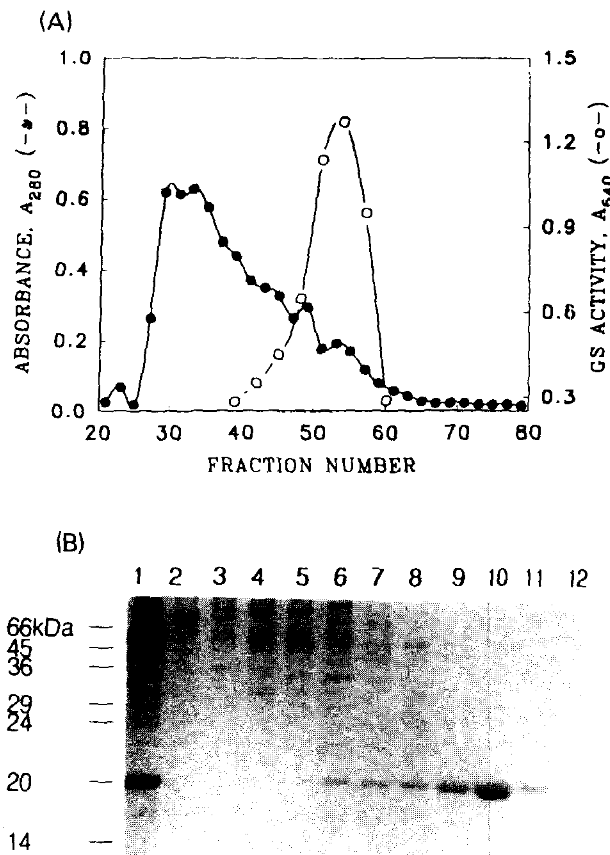

Fig. 2. Purification of *E. coli* thiol peroxidase.

(A) Fractionation of IPTG-induced *E. coli* BL21 (pSK-TPX) osmotic shock fluid by gel permeation chromatography. Approximately 40 mg of ammonium sulfate precipitated proteins was loaded onto a Sephacryl S200 HR column (2.5×45 cm). The protein concentration was monitored by absorbance at 280 nm. Fractions were collected as 3 ml each. The antioxidant activity of thiol peroxidase was measured by assaying 10 μl of eluted proteins for the presence of *E. coli* glutamine synthetase (GS) protection activity against DTT/Fe^{3+} oxidizing system [11]. (B) SDS-PAGE analysis of thiol peroxidase fractions separated by gel permeation chromatography. Protein samples were electrophoresed in 12% SDS-PAGE and visualized by Coomassie Brilliant Blue R-250. Lane 1, 15 μg of osmotic shock fluid protein from *E. coli* BL21 (pSK-TPX); lanes 2~12, 10 μl of sample from fraction numbers 30 to 60.

To evaluate the identity of the new 20-kDa protein synthesized by *E. coli* BL21 cells containing the pSK-TPX recombinant plasmid, the overproduced protein was isolated and its antioxidant activity was measured. For large scale purification of overproduced thiol peroxidase from periplasm we used cold osmotic shock [8] for *E. coli* BL21 carrying the pSK-TPX plasmid. Periplasmic proteins in the cold osmotic shock fluid were precipitated by ammonium sulfate at 80% saturation, and the precipitated proteins were loaded onto a Sephacryl S-200 HR column. The antioxidant activity of eluted proteins functioned to protect against oxidative damage of *E. coli* glutamine synthetase by the DTT/Fe^{3+} oxidizing system [11]. Figure 2 shows that antioxidant activities were correlated with the intensities of 20-kDa bands on SDS-PAGE. The column fraction that exhibited the greatest antioxidant activity revealed a homogenous band on SDS-PAGE analysis (Fig. 2B, lane 10). The purified protein showed the same antioxidant activity and the same molecular weight on SDS-PAGE as the authentic enzyme from wild type *E. coli* K12 (data not shown). On average, 20 to 30 mg of homogeneous thiol peroxidase was routinely obtained from 1 g of cell pellet (wet weight).

In conclusion, the thiol peroxidase expression system using a T7 RNA polymerase/promoter system described here should provide a convenient method for purification of this biologically active protein.

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