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The *Escherichia coli btuE* gene, encodes a glutathione peroxidase that is induced under oxidative stress conditions

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ABSTRACT

Most aerobic organisms are exposed to oxidative stress. Looking for enzyme activities involved in the bacterial response to this kind of stress, we focused on the *btuE*-encoded *Escherichia coli* BtuE, an enzyme that shares homology with the glutathione peroxidase (GPX) family. This work deals with the purification and characterization of the *btuE* gene product.

Purified BtuE decomposes *in vitro* hydrogen peroxide in a glutathione-dependent manner. BtuE also utilizes preferentially thioredoxin A to decompose hydrogen peroxide as well as cumene-, tert-butyl-, and linoleic acid hydroperoxides, confirming that its active site confers non-specific peroxidase activity. These data suggest that the enzyme may have one or more organic hydroperoxide as its physiological substrate.

The *btuE* gene was induced when cells were exposed to oxidative stress elicitors that included potassium tellurite, menadione and hydrogen peroxide, among others, suggesting that BtuE could participate in the *E. coli* response to reactive oxygen species. To our knowledge, this is the first report describing a glutathione peroxidase in *E. coli*.

1. Introduction

Aerobic organisms have evolved a number of enzymatic and non-enzymatic antioxidant defense systems which function in a cooperative manner to protect the cell from oxidative stress [1]. Examples of enzymatic antioxidant mechanisms include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). In eukaryotic organisms, GPXs are one of the major enzymatic defenses against hydroperoxides and/or membrane lipid peroxidation [2]. However, little is known about prokaryotic glutathione peroxidases. Exceptions are *Neisseria meningitidis* and *Streptococcus pyogenes*, where it has been shown that GPXs are involved in defense against oxidative stress [3–6]. In general, GPXs catalyze the decomposition of organic hydroperoxides (ROOH) and hydrogen peroxide (H_2O_2) according to the reaction [7,8]:

$$ROOH + 2GSH \rightarrow ROH + GSSG + H_2O$$

where GSH and GSSG denote reduced and oxidized glutathione, respectively.

The *Escherichia coli* genome displays an open reading frame exhibiting striking similarity to other genes encoding glutathione peroxidases [9]. This gene, *btuE*, lies within the *btuCED* operon, involved in vitamin B12 transport [10,11]. In spite of its genetic neighborhood, *btuE* seems not to be involved in B12 transport; in fact, deletions up to 82% of *btuE* does not affect B12 transport in *E. coli* [12], which actually depends on *E. coli btuC*, *btuD* and *btuF* genes [13,14].

To date, available evidence about BtuE function is merely theoretical and suggests that this enzyme, a putative member of the phospholipid glutathione peroxidase family [15], could function as a selenium-independent GPX [16]. The 552 bp *E. coli btuE* gene encodes a protein of 183 amino acid residues with a *Mr* of 20 kDa. Like most GPXs, BtuE contains the conserved Cys, Trp and Gln residues at the active site. Although exhibiting a putative oligomerization interface, the lack of the tetramerization "PGGG" motif predicts a homodimeric structure for BtuE [9].

In this work, we characterized biochemically the *btuE* gene product and found that BtuE catalyzes the decomposition of a variety of peroxides in the presence of thioredoxin A or C as the reducing agent, confirming that its active site confers non-specific peroxidase activity. We also found that *btuE* expression was induced under oxidative stress conditions and that it is paralleled by an increased BtuE synthesis.

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2. Materials and methods

2.1. Bacteria and culture conditions

Bacteria were grown routinely in LB medium [17] at 37 °C with shaking. Growth was initiated by inoculating fresh LB medium with 1:100 dilutions of overnight cultures. Solid media contained 2% (w/v) agar and plates were incubated overnight at 37 °C.

The *E. coli btuE*::*lacZ* strain was constructed using *E. coli* BW25113 Δ*btuE (btuE::kan*, NARA Institute, Japan) and plasmids pCP20 and pCE37, essentially as described [18]. Integration and correct orientation of pCE37 in the host chromosome was analyzed by PCR using primer pairs TGTAGGCTGGAGCTGCTTCG/GACCATTTTCAATCCGCA and CATATGAATATCCTCCTTAG/TTTCTAGAGC TGTTAAAAGGACA. In addition, the absence of multiple integrations was checked by PCR using primers GACCATTTTCAATCCGCA and TTTCTAGAGCTGTTAAAAGGACA.

2.2. Protein purification

BtuE and BCP (Bacterioferritin Comigratory Protein) [19] peroxidases as well as GorA, GrxA, GrxB, GrxC, TrxA, TrxB and TrxC gene products were purified from *E. coli* AG1 carrying the respective structural genes cloned into the pCA24 N vector (ASKA Collection, Nara Institute, Japan). Cells were grown to an OD_{600} ~0.5 and induced with 1 mM IPTG for 5 h. Crude extracts were prepared in 20 mM sodium phosphate buffer, pH 7.4, that contained 0.5 M NaCl and 20 mM imidazol (buffer A). After washing exhaustively with the same buffer, proteins were purified by HisTrap HP (Amersham) affinity column chromatography as recommended by the vendor. Protein concentration was determined as previously described [20].

2.3. Determination of peroxidase activity

BtuE and BCP were tested for peroxidase activity essentially as described previously [19], except that H_2O_2, cumene- and tert-butyl hydroperoxide concentrations used were 450 μM instead of 700 μM.

To determine its reducing substrates, BtuE was assayed at 25 °C in a mixture (1 ml) that contained 10 mM potassium phosphate buffer, pH 7.0, 250 μM NADPH, 1 mM EDTA, 1 mM GSH (or 3 μM glutaredoxin or thioredoxin), 0.1 μM GR or TR (as required) and 0.5 mM H_2O_2, CUOOH or t-BOOH. LAOOH was tested at 0.1 mM in the presence of 0.1% Triton 20. Reactions were started by adding the enzyme (8 μM) and absorbance was recorded at 340 nm. Blanks of NADPH oxidation before enzyme addition were subtracted from the calculations.

2.4. RNA purification and qRT-PCR

Total RNA was purified utilizing the QIAGEN RNAsy kit. Briefly, *E. coli* BW25113 cells were inoculated in 200-ml Erlenmeyer flasks and incubated at 37 °C with shaking to an OD_{600} ~0.5. Cultures were then supplemented with either 0.5 μg/ml K_2TeO_3 (final concentration) or 100 μM H_2O_2. After 20 min, cells were sedimented at 13,000×*g* for 3 min, washed and RNA was purified, treated with RNAse-free RQ1 DNAse and quantified using the Quant-iT RiboGreen® RNA (Invitrogen) following the vendor instructions. Real-time RT–PCR experiments were performed with a LightCycler® RNA Amplification Kit SYBR Green I (Roche Applied Science) using approximately 2 μg of RNA as described previously [21].

3. Results and discussion

Although the *E. coli* BtuE protein was suspected to function as a glutathione peroxidase [15], the experimental evidence was missing. In this context, the aim of this work was to purify BtuE from this bacterium and to characterize its peroxidase activity. BtuE was purified near to homogeneity by affinity chromatography as described in Section 2; BCP peroxidase was purified in parallel and used as positive control for peroxidase activity (Fig. S1). Since BtuE reducing substrates were not known, in a first attempt we used dithiotreitol as reducing agent. Fig. 1 shows that BtuE decomposes hydrogen peroxide, cumene-, tert-butyl hydroperoxide and linoleic acid peroxide, indicating that the enzyme's active site confers non-specific peroxidase activity. BtuE showed lower activity

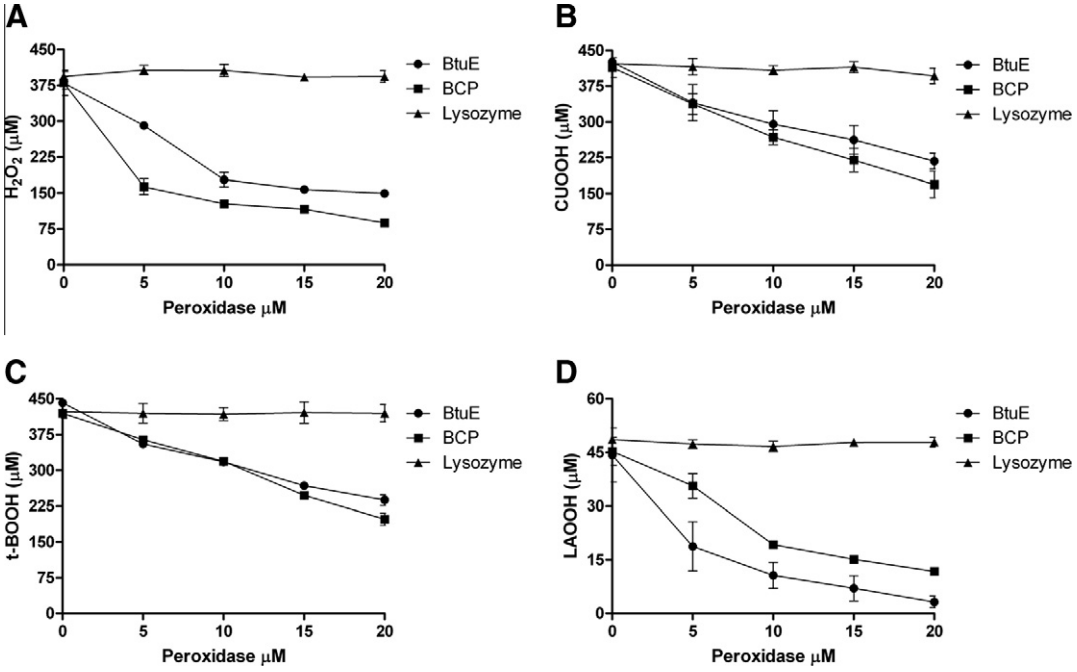

Fig. 1. Peroxidase activity of BtuE *in vitro*. The enzyme was reacted with hydrogen peroxide (A), cumene (B), tert-butyl hydroperoxide (C) and linoleic acid peroxide (D) as described in Section 2. BCP and lysozyme were used as positive and negative controls, respectively. Bars represent standard deviation ($n = 3$).

than BCP regarding hydrogen peroxide, while both peroxidases showed similar activity with cumene- and tert-butil hydroperoxide. In turn, BtuE was more active than BCP with linoleic acid hydroperoxide (Fig. 1), suggesting that lipid peroxides could be the real, physiological substrates of BtuE. These results are in agreement with the peroxidase activity exhibited by Se-independent peroxidases with lipid peroxides [16,22,23] and suggest that BtuE could protect the cell against the harmful effects of these compounds.

On the other hand, and given that some GPXs also use thioredoxins or glutaredoxins as reducing substrates [24,25], glutathione and thioredoxin reductases (GorA and TrxB, respectively) as well as glutaredoxins GrxA, GrxB, GrxC and thioredoxins A and C were purified to test if they function as reducing substrates for BtuE peroxidase. Substrate specificity was analyzed using the same peroxides as above. Fig. 2 shows that BtuE uses preferentially thioredoxin A or C as reducing agents irrespective of the peroxide substrate.

To test if BtuE peroxidase was related to oxidative stress, an *E. coli* harboring the *lacZ* gene under the control of the *btuE* promoter was constructed as described in Section 2. The expression of the *btuE* gene was then analyzed in different growth conditions and in the presence of different oxidative stress elicitors. Fig. 3A shows increased *btuE* expression levels in the stationary growth phase, most probably as consequence of higher oxidative stress levels occurring during this growth stage [1]. On the other hand, and to determine if *btuE* expression was induced by oxidative stress generators, β-galactosidase activity was assessed in cells exposed to potassium tellurite (K_2TeO_3), menadione, H_2O_2, CUOOH, t-BOOH, diamide or low temperature (15 °C). As seen in Fig. 3B, an important induction of *btuE* expression was observed in cells exposed to the superoxide inducers tellurite [24,26–29] and menadione [30] or hydrogen peroxide.

Next, *btuE* transcriptional induction was evaluated in response to K_2TeO_3 or H_2O_2 by qRT-PCR using total RNA from *E. coli* BW25113—

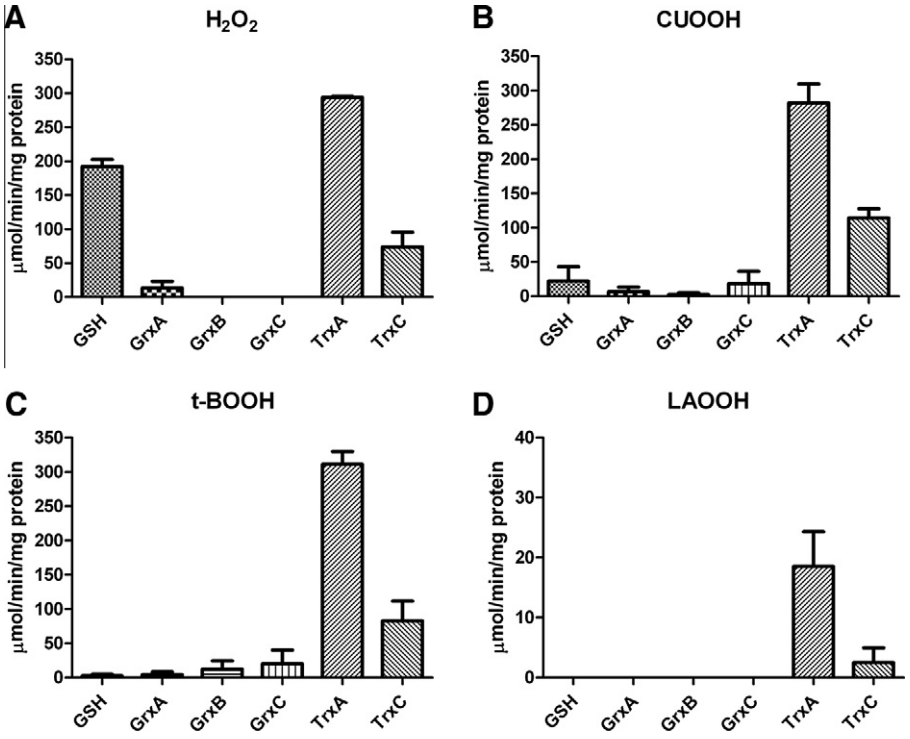

Fig. 2. Reducing substrates for BtuE *in vitro*. The enzyme was reacted with hydrogen peroxide (A), cumene (B), tert-butyl hydroperoxide (C) and linoleic acid peroxide (D) as described in Section 2 in the presence of the indicated reducing substrates. Bars represent standard deviation ($n = 4$).

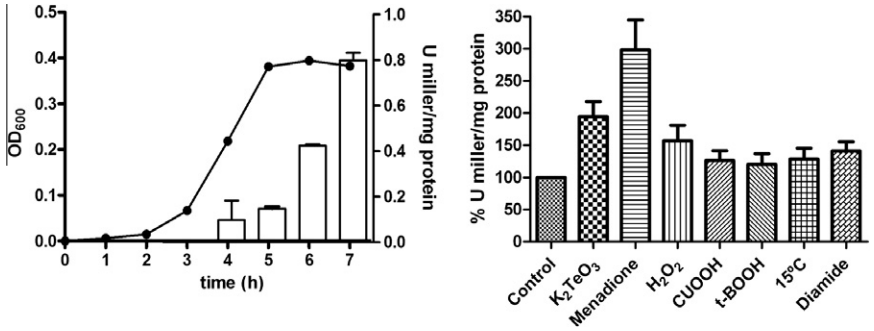

Fig. 3. Induction of *btuE* in response to oxidative stress conditions. (A) β-Galactosidase activity (white bars) was determined as described previously [31] for the *btuE*::*lacZ* reporter strain along the growth curve. (B) *btuE* Transcriptional levels determined after exposing *E. coli btuE*::*lacZ* for 20 min to K_2TeO_3 (0.5 µg/ml), menadione (500 µM), H_2O_2 (100 µM), CUOOH (100 µM), t-BOOH (100 µM), low temperature (15 °C) or diamide (2 mM). 100% β-Galactosidase activity was that of control, untreated cells. Bars represent standard deviation ($n = 4$).

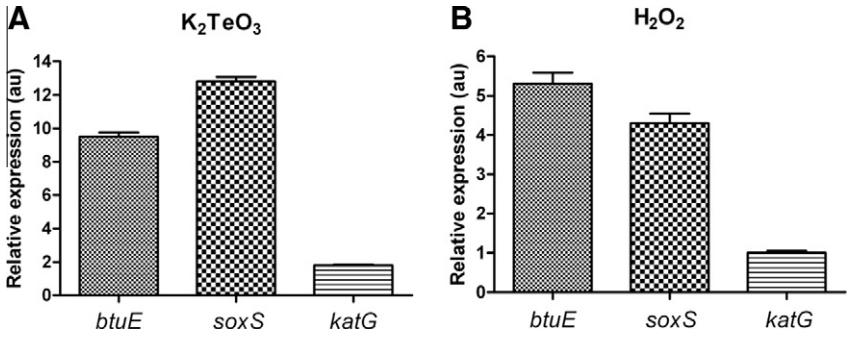

Fig. 4. Relative *btuE* expression in *E. coli* exposed to tellurite or hydrogen peroxide. Transcriptional levels of *btuE*, *soxS*, *katG* or *rpoD* were determined as described earlier [21] after exposing wild type *E. coli* BW25113 for 20 min to K_2TeO_3 (0.5 μg/ml) or H_2O_2 (100 μM). Relative expression was calculated regarding that of the housekeeping *rpoD* gene. Bars represent standard deviation ($n = 4$).

exposed or not to these toxicants—as template. Crossing point values were determined as previously described [21] and the relative expression of *btuE*, *soxS* and *katG* genes regarding that of the housekeeping control, the *rpoD* gene, was determined. Fig. 4 shows that *btuE* and *soxS* transcription is heavily induced in the presence of both tellurite and hydrogen peroxide, again suggesting the participation of BtuE in the *E. coli* response to oxidative stress. The increased transcription was paralleled by an increase of BtuE protein concentration in cells exposed to these toxicants (Fig. S2).

Given the observed induction of *btuE* under oxidative stress conditions, the promoter sequence of the *btuE* gene was analyzed looking for putative transcriptional regulator binding boxes. As seen in Fig. S3, a putative box for the global regulator of the response against hydrogen peroxide, OxyR, was found which may explain in part the observed transcriptional activation mentioned above. Experiments to analyze in more depth this issue are being carried out at our laboratory.

Summarizing, our interest in bacterial aerobic metabolism and the dearth of knowledge regarding prokaryotic GPXs prompted us to analyze the role of the *E. coli btuE* gene product. Results from this work represent a step forward in our current knowledge about the *E. coli* BtuE enzyme function and suggest that this enzyme could protect against the deleterious effects of various oxidants, making the cell less sensitive to oxidative stress. This protective effect could be strengthened given the ability of BtuE to use more that one reducing agent. In this sense, preliminary results of our group indicate that *btuE* elimination results in profound consequences *in vivo*, confirming the importance of this enzyme. Additional experiments to unveil the real function of BtuE and/or its specific mechanism of action are under way in our laboratory.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.07.002.

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