Alkali- and Halo-tolerant Catalase from \textit{Halomonas} sp. SK1: Overexpression in \textit{Escherichia coli}, Purification, Characterization, and Genetic Modification

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A catalase gene, \textit{ohktA}, from an alkali- and halo-tolerant bacterium, \textit{Halomonas} sp. SK1, on the pKK223-3, was expressed in the catalase-lacking \textit{Escherichia coli} strain UM2. Highly purified catalase showing a single band on SDS-PAGE was obtained by two liquid chromatography steps on DEAE-Toyopearl \textsuperscript{1} and Chelating-Sepharose Fast Flow. The enzyme, oHktA, shows high catalase activity with a pH optimum at 10, and the activity was stable in 4M KC\textsubscript{1}l. This enzyme is thermo-sensitive, showing a significant loss of activity within 5 minutes at 37°C. To modify the stability of the catalase, the addition of domain II of the heat stable Mn catalase from \textit{Thermus thermophilus} to the C-terminus was made. When coexpressed with a chaperone (PhFKBP29) gene product, peptidyl–prolyl \textit{cis}–\textit{trans} isomerase, from a thermophilic bacterium, a chimeric catalase was produced in the soluble fraction. The stability of this catalase in the range of 37°C–45°C was improved and it was stable for more than 1 h at 37°C.

\textbf{Key words:} alkali- and halo-tolerance; catalase; \textit{Halomonas}; overexpression in \textit{Escherichia coli} UM2; chaperone

Catalase (EC1.11.1.6) is an antioxidant enzyme in the cellular protection system; it decomposes H\textsubscript{2}O\textsubscript{2} to water and oxygen.\textsuperscript{1} Catalase comprises three subgroups, in which the largest one, typical catalases are 200–340 kDa, homotetramers with four prosthetic groups; they are distributed in most respiring organisms.\textsuperscript{2,5}

In our previous report, oHktA, an alkali- and halo-tolerant catalase from \textit{Halomonas} sp. SK1 living in an oxidative environment, was purified and characterized. Furthermore, its gene was cloned and sequenced. From these data, the oHktA was identified as a typical catalase.\textsuperscript{3,5}

Some catalases have elongated C-terminal domains which obviously contribute to increase stability against chemicals and under high temperature.\textsuperscript{2,3} PVC and HPII, typical catalases from \textit{Penicillium vitale} and \textit{Escherichia coli}, respectively, contain such domains with a “flavodoxin-like” topology.\textsuperscript{2,3} Mn catalases from extremophilic bacteria\textsuperscript{4–6} and thermostable catalase-peroxidase from \textit{Bacillus stearothermophilus}\textsuperscript{7} also have such domains. Especially in domain II of the Mn catalases from extremophiles, the helical contents are higher than those from mesophiles.\textsuperscript{4,6}

In this report, the construction of an overproduction plasmid for oHktA is described. By placing the \textit{hktA} gene under the tac promoter, abundant quantities of enzyme were obtained in a soluble form. The expression and characterization of the chimeric catalase cHktA, produced by the fusion of Mn catalase domain II from \textit{Thermus thermophilus} to the C-terminus of oHktA, are also described.

\textbf{Materials and Methods}

\textit{Bacterial strains and plasmids.} \textit{Halomonas} sp. SK1 was cultured as reported previously.\textsuperscript{3,5} The catalase-lacking \textit{Escherichia coli} strain UM2, kindly provided by Prof. C. P. Loewen, was used as a source of host cells for
the expression of catalase genes on the pKK223-3 plasmid (Amersham Pharmacia Biotech, USA). This strain lacks catalase activity due to mutations in the catalase genes (both katE and katG). It was cultivated in 2xYT medium (pH 11.0) at 37°C under aerobic conditions for 8–16 h. Thermus thermophilus HB8 (ATCC27634) was purchased from National Collection of Industrial and Marine Bacteria (NCIWB), Japan. It was grown in TY salts medium at 60°C under aerobic conditions for 5–8 d. Pyrococcus horikoshii was obtained from the Japan Collection of Microorganisms (Riken, Saitama, Japan). The culture of this bacterium and the construction of the expression plasmid pACPhFK-1 were reported previously.\(^9\)

**Construction of the expression plasmid for the OktA.** The gDNA of Halomonas sp. SK1 cells was isolated by a standard phenol–chloroform method\(^10\) and used as the template for PCR reactions using primers hkt1 and hkt2 with EcoRI and HindIII recognition sites (underlined letters), respectively. The hkt1 (5’-CCG GAA TTC CAT GAG CGA CGA CGA CAC GAA AAAA GT-3’) and hkt2 (5’-TTA AAG TTC TTA CGG AGG CAG GGC GAC CAG-3’) primers contain initiation and termination codons (bold letters), respectively. The PCR product, digested with EcoRI and HindIII, was purified and ligated into the pKK223-3 plasmid, followed by the transformation into E. coli UM2 cells and the isolation of positive clones on 2xYT (pH 7.4) agar plates containing 100 µg/ml ampicillin. The constructed expression vector for the original catalase was named pKK223-3-ohkt.

**Purification of the catalase and analysis of its activity.** For the catalase, purification, analysis of the protein component by the SDS-PAGE or native-PAGE, measurement of the activity and protein amounts, and the characterization were carried out as previously described.\(^3\) One liter of 2xYT-broth (pH 7.4) supplemented with 100 µg/ml ampicillin was inoculated with 10 ml of E. coli HB8 and incubated for conditions for 8–16 h. Thermus thermophilus HB8 (ATCC27634) was purchased from National Collection of Industrial and Marine Bacteria (NCIWB), Japan. It was grown in TY salts medium at 60°C under aerobic conditions for 5–8 d. Pyrococcus horikoshii was obtained from the Japan Collection of Microorganisms (Riken, Saitama, Japan). The culture of this bacterium and the construction of the expression plasmid pACPhFK-1 were reported previously.\(^9\)

**Construction of an expression plasmid for a chimeric catalase, cHktA.** To construct the chimeric catalase gene, chktA, two PCR reactions were done. In the first PCR, two primers, hkt1 and hkt3 (5’-GCC GCA GAT ACG CAG GCA GGG CGA CCA GTG GG-3’) with a PsI recognition site, and gDNA of Halomonas sp. SK1 were used. In the second PCR, two primers, hkt4 (5’- CCG GTG ATT GGC GAT-3’) with a PsI recognition site and hkt5 (5’- CCC AAG ATT TTA TTT TGC TTT TTC ATA TAA TTT CTT GGC GAT-3’) with a HindIII recognition site, and gDNA of Thermus thermophilus were used. The recognition sites in each primer are shown by underlined letters. Then, two PCR products were digested with PsI, purified and ligated to obtain chktA. Finally, chktA and the pKK223-3 plasmid were digested with EcoRI and HindIII, purified and then ligated, forming a chimeric catalase expression vector (pKK223-chktA). Another plasmid, pACPhFK-1, carrying a T7 promoter and an archaeal gene, PhFKBP29, was prepared as described by Ideno et al.\(^9\) The PCR amplified DNA fragment for PhFKBP29 was digested with NdeI and HindIII and then ligated into pET21a (Novagen), resulting in the formation of expression vector pEPhFK-1. Also, the PhFKBP29 gene, together with the adjacent T7 promoter and terminator in pEPhFK-1, was PCR amplified and the DNA fragment obtained was digested with SphI and SalI, followed by the ligation into the plasmid vector, pACYC184 (Nippon Gene, Tokyo, Japan). The resulting expression vector for PhFKBP29 was named pACPhFK-1. E. coli UM2 cells harboring both plasmids, pACPhFK-1 and pKK223-3-chktA, were obtained on 2xYT (pH 7.4) agar plates supplemented with 100 µg/ml each of ampicillin and chloramphenicol.

**Results and Discussion**

**Production, purification, and characterization of OktA.**

By the PCR using primers hkt1 and hkt2, a DNA fragment of 1,530 bp, corresponding to the entire sequence of ohktA, was obtained. Placement of this fragment under the tac promoter in the pKK223-3 plasmid resulted in abundant production of soluble enzyme in E. coli UM2 catalase lacking cells. The enzyme, OktA, was observed on SDS-PAGE as a predominant band at a position corresponding to 57.8 kDa, consistent with the molecular mass calculated
from the deduced amino acid sequence (Fig. 1).

As in the case of the Halomonas sp. SK1 wild-type catalase HktA, purification of this catalase, oHktA, was accomplished by chromatography on DEAE-Toyopearl and Chelating-Sepharose Fast Flow columns (Fig. 1) with 18.8-fold purification at 93.5% yield (Table 1). The purified oHktA showed a high specific activity of 80,700 U/mg of protein. In native-PAGE, a protein band with catalase activity was detected at the position corresponding to the BLC activity signal (Fig. 2A, B).

The recombinant oHktA showed following characteristics (data not shown), similar to the wild-type enzyme, HktA. Approximately 90% of its activity remained in the presence of 15 mM dithionite (DT), but the activity was completely lost in the presence of 15 mM 3-amino-1,2,4-triazole (AT). In addition, the relative oHktA activity was reduced to approximately 50% in 20 μM NaN₃, 80 μM NH₂OH, or 100 μM KCN. The highest activity was detected at pH 10.0. The remaining activity levels at pH 4.0 and pH 12.0 were 21% and 14%, respectively. The KCl concentration that produced optimal oHktA activity was broad (0 to 1.5 M), with 74% activity remaining at 4 M KCl, 3 times higher than that of BLC. The catalase oHktA is thermo-sensitive. Although no reduction in activity during 3 h at 25 °C was observed, the activity was significantly reduced following 5 min at 37 °C, 40 °C, or 55 °C.

For most industrial applications, especially in the textile industry, bovine liver catalase is used. In this case, oHktA, cloned from a bacterium that survives under extreme conditions in the waste water from a textile factory, may be a suitable replacement as it shows high activity under alkaline and high salt conditions.

Table 1. Summary of the Purification of the Original Alkali- and Halo-tolerant Catalase, oHktA, from Overexpressed E. coli Cells

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg of protein)</th>
<th>Yield (%)</th>
<th>Purification (Fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>146</td>
<td>63.0 × 10⁴</td>
<td>4.30 × 10⁴</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>DEAE-Toyopearl</td>
<td>27.3</td>
<td>60.9 × 10⁴</td>
<td>2.23 × 10⁴</td>
<td>96.7</td>
<td>5.20</td>
</tr>
<tr>
<td>Chelating-Sepharose Fast Flow</td>
<td>7.30</td>
<td>58.9 × 10⁴</td>
<td>8.07 × 10⁴</td>
<td>93.5</td>
<td>18.8</td>
</tr>
</tbody>
</table>

One unit (U) of activity is defined as the amount of enzyme that decomposes 1 μmole of H₂O₂ per min.

Fig. 1. SDS-PAGE Analysis of the Expression of oHktA in E. coli UM2 Cells and Its Purification.

Lane 1, low molecular weight markers; Lanes 2 and 5, proteins in the supernatant fraction (10.0 μg protein/lane) and precipitate fraction (12.0 μg protein/lane), respectively, of E. coli UM2/pKK223-3-ohktA (with IPTG induction); Lane 3, total cell proteins (12.0 μg protein/lane) from E. coli UM2/pKK223-3-ohktA (without IPTG induction); Lane 4, total cell proteins (12.0 μg protein/lane) from E. coli UM2/pKK223-3; Lane 6, flow-through fraction from a DEAE-Toyopearl column (9.0 μg protein/lane); Lane 7, the fraction eluted with 10 mM imidazole from the Chelating-Sepharose Fast Flow column (3.2 μg protein/lane). The positions of low molecular weight markers are shown on the left. The arrow (on the right) indicates the position of oHktA.

Fig. 2. Native-PAGE Analysis of the Crude Extract Proteins and Purified oHktA from E. coli UM2/pKK223-3-ohktA.

(A) CBB staining. Lane 1, high molecular weight markers; Lane 2, crude extract (30.0 μg protein/lane); Lane 3, fraction eluted with 10 mM imidazole from a Chelating-Sepharose Fast Flow column (3.0 μg protein/lane). The positions of high molecular weight markers, including 232 kDa bovine liver catalase (BLC), are shown on the left. (B) Staining for catalase activity. Lane 1, high molecular weight markers, including 232 kDa bovine liver catalase (BLC); Lane 2, crude extract (0.5 μg protein/lane); Lane 3, fraction eluted with 10 mM imidazole from a Chelating-Sepharose Fast Flow column (0.06 μg protein/lane). The arrow (on the right) indicates oHktA.
Fig. 3. Alignment of the Amino Acid Sequence of the oHktA with Those of the Chimeric Alkali- and Halo-tolerant Catalase (cHktA), and Catalases from *Vibrio rumoiensis* S-1 (VktA), *E. coli* (HP II), and *Penicillium janthinellum* (PVC).

Multiple alignments were carried out using the CLUSTAL W program. Residues that are identical in all five catalases are shaded, and distinct domains are indicated by horizontal bars. The longer C-terminal and C11-helical structure in cHktA, as estimated by the 3D-PSSM program, are underlined and boxed, respectively.
Expression of the chimeric catalase cHktA, and its refolding by a chaperone from a thermophilic bacterium

To construct a chimeric catalase, the ohktA was connected to a DNA fragment corresponding to the C-terminal domain II of the thermo-stable catalase from Thermus thermophilus. This domain (Gly201 to Lys302) with 46% hydrophobic amino acids is predicted to form an α-helical structure (Fig. 3) and to work as a strong protecting wrapper for the active site.2,4–7) The chimeric gene, chktA, was overexpressed in E. coli UM2 cells by IPTG induction. Most of the recombinant cHktA protein appeared in the insoluble fraction as inclusion bodies without activity. However, coexpression with a chaperone, PhFKBP29, from a thermophilic bacterium, succeeded in producing cHktA as a soluble protein. In both cases, the apparent molecular weight of the cHktA on SDS-PAGE was 62.4 kDa (data not shown). Because the chktA gene was confirmed by DNA sequencing, that anomalous protein immigration occurred in the SDS-PAGE may be arrived from the protein structure and the amino acid composition of the additional C-terminus.

The PhFKBP29 chaperone enhanced the soluble expression of Fab by refolding this protein, interacting with the hydrophobic region.10) A similar mechanism may also operate in the refolding of cHktA.

For the cHktA, the absorption spectrum, heme content and all other characteristics, including an optimum activity at pH 10 and halo tolerance, were indistinguishable from those of the original oHktA (data not shown). However, the chimeric catalase is more thermo-stable than the original catalase, oHktA. As shown in Fig. 4, stability in the range of 37–45°C was improved. Although the activity of cHktA was suppressed at 40–60°C, it was retained for more than 1 h at 37°C (data not shown). At 37°C, the specific activities (U/mg of protein) for oHktA and cHktA were 3.52 × 10⁴ and 6.51 × 10⁴, respectively. In the tertiary-structures predicted by the 3D-PSSM program15) for oHktA and cHktA, their sequence-structure compatibilities are similar to that of HPII. The presence of stable α-helices in cHktA formed by the additional C-terminus and its docking onto the surface of the β-barrel domain (catalytic core) were predicted (Fig. 5). Finally, it is suggested that the elongated C-terminal peptide enhances the thermo-stability of this catalase. The data also suggest an influence of the C-terminal domain on the folding of the catalytic core of the catalase.

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