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Catalase of *Staphylococcus warneri* ISK–1 Isolated from *Nukadoko*

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An intracellular catalase from *Staphylococcus warneri* ISK–1 was purified to homogeneity in a six–step purification procedure. The purification of catalase, as judged by the final specific activity of 10,800 U mg^{-1}, was 310–fold with a 14% yield. The native enzyme had a molecular weight of 125,000 and was composed of two subunits of equal size (64,000). The absorption spectrum of the catalase showed a soret band at 406 nm, indicating that the enzyme is a heme protein. As a result of the determination of various inhibitors on the catalase activity, ISK–1 catalase was a typical monofunctional catalase. The specific activity throughout the growth of batch culture with or without aeration was investigated and three–fold elevated activity was found in the aerobic culture.

INTRODUCTION

Bacteria have specific enzymes to detoxify potentially lethal reactive oxygen spieces (ROS), including superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$). The effects of ROS result in the oxidation of various cellular components such as DNA, RNA, proteins and lipids. Therefore, bacteria maintain low level of ROS concentrations during normal aerobic respiration. For example, the concentrations of the intracellular $O_2^{\cdot-}$ and H_2O_2 for aerobically growing *Escherichia coli* cells have been measured to be around 10^{-10} M and 10^{-7} M, respectively (Gonzalez–Flecha and Demple, 1995; Imlay and Fridovich, 1991). There are, however, various environmental agents such as UV–radiation and numerous compounds (*e. g.* paraquat, plumbagin, and menadione) that generate intracellular $O_2^{\cdot-}$ (Sies, 1991; Kappus and Sies, 1981). In the meantime, phagocytes also produce ROS for the defence against microorganisms. Phagocytes use an NADPH oxidase to produce $O_2^{\cdot-}$, which reacts to produce H_2O_2 and $HO^{\cdot}$ (Clark, 1990; Hassett and Cohen, 1989; Johnson and Kitagawa, 1985; Weiss and LoBuglio, 1982). In spite of the host defence response, many pathogenic bacteria have been shown to enable to survive in phagocytic cells (Casey *et al.*, 1986; Parsons *et al.*, 1981; Morris *et al.*, 1992). Thus bacterial defence mechanisms against oxidative stress play a very important role for their survival.

Catalase is one of the central defence enzymes against oxidative stress, which catalyzes the conversion of H_2O_2 to water and molecular oxygen. Many bacteria possess two types of catalases, typical catalase (monofunctional catalase) which catalyzes H_2O_2 only; catalase–peroxidase which also has an associated peroxidase activity. They are distinct from each other in many properties. Typical catalase is composed of four 60 kDa

subunits, containing 2.5–4 heamin prosthetic groups per tetramer and showing a broad optimum pH in the range of 5–10. It is resistant to treatment with ethanol/chloroform and is specifically inhibited by 3-amino-1,2,4-triazol (Margoliash *et al.*, 1960). Catalase-peroxidase having an optimum pH at 6–6.5, is relatively more sensitive to temperature, ethanol/chloroform than the typical catalases, but it is insensitive to 3-amino-1,2,4-triazol (Goldberg and Hochman, 1989b; Hochman *et al.*, 1992).

The genus *Staphylococcus* comprises a group of gram-positive facultative anaerobic bacteria which include the opportunistic human pathogen *S. aureus* (Waldvogel, 1985). Methicillin-resistant strains of *S. aureus* have also appeared with the recent increase in antibiotic resistance. This has highlighted the importance of investigating the stress response in pathogenic bacteria as a clue to elucidating the mechanisms of resistance to antibiotic and host defence reaction, and of virulence expression. However, little is known about staphylococcal catalase itself, although the catalase activity has been shown to be involved in ability to survive during phagocytosis in *S. aureus* (Leijh *et al.*, 1980, 1981).

In this report, we purified and characterized a catalase from *S. warneri* ISK-1 isolated from well aged *Nukadoko* that is the rice bran packed fermentation bed for Japanese traditional pickled vegetables (Herawati and Ishizaki, 1997). This bacterium was identified as a strain of *S. warneri* from its physiological and biochemical characteristics, analysis of its 16 S rRNA sequence, and DNA-DNA relatedness.

MATERIALS AND METHODS

Organism and culture conditions

S. warneri ISK-1 was isolated from well aged *Nukadoko* (Herawati and Ishizaki, 1997). Cultures were grown at 37 °C on an orbital shaker at 200 rpm in 500-ml Sakaguchi-flasks containing 200 ml MRS (Oxoid Co., Hampshire, England) medium. Ten ml starter cultures were grown for 18 h in TGC (Difco Laboratories Co., Detroit, MI, USA) medium and then used to inoculate 200 ml pre-cultures. These cultures were then used to inoculate 4×200 ml cultures (10 ml inoculum per 200 ml), which were grown for a further 8 h and harvested at late-exponential growth phase. For monitoring of catalase activity at different times throughout the growth, jar fermentations under aerobic and microaerobic conditions were carried out. The cultures were microaerobically grown at 37 °C and 440 rpm in 1-l jar fermentor containing 500 ml MRS medium. Aerobic cultivation was performed by aeration at a rate of 0.4 vvm.

Catalase assay

Catalase was assayed by following the disappearance of H_2O_2 (Santoku Chemical Industries Co., Tokyo) spectrophotometrically at 240 nm as reported previously (Beer and Sizer, 1952). The assay cocktail contained 50 mM potassium-phosphate buffer (pH 7.0) and 20 mM H_2O_2 in a final volume of 3 ml. One unit of activity was defined as the amount of enzyme catalyzing the degradation of 1 μmol H_2O_2 per min at 25 °C.

Protein estimation

Protein was determined by the method of Bradford with bovine serum albumin

(Bio-Rad, Hercules, CA, USA) as a standard (Bradford, 1976).

Purification of catalase

Unless otherwise stated, all the following manipulations were done at 4°C. All columns used were equilibriated with 50 mM potassium-phosphate buffer (pH 7.0).

Step 1: Extraction and centrifugation

The cells from 1 l of culture were harvested by centrifugation at 5,500×g for 15 min, washed twice with 50 mM potassium-phosphate buffer (pH 7.0), and suspended in 30 ml of the buffer. The cells were then disrupted by sonication with a Cell Disruptor 350 (Branson Sonic Power Co., Danbury, CT, USA) at 50% duty cycle and followed by cooling for 30 sec to prevent excessive heating. The total time of the treatment was 20 min. The cell suspension sonicated was then centrifuged at 23,000×g for 30 min and catalase was purified from the resulting cell-free extract.

Step 2: Streptomycin sulfate precipitation

Solid streptomycin sulfate (Nacalai Tesque Co., Kyoto) was added to give 100% (w/w) of total protein to remove DNA and RNA. The mixture was stirred for 20 min and the precipitate was removed by centrifugation at 18,500×g for 10 min.

Step 3: Ammonium sulfate precipitation

The supernatant was then adjusted to 70% saturation with solid ammonium sulfate and stirred for 20 min. The precipitated proteins were collected by centrifugation at 18,500×g for 10 min, dissolved in 2 ml of 50 mM potassium-phosphate buffer (pH 7.0) and dialyzed overnight against the buffer.

Step 4: Anion-exchange chromatography on DEAE-Sephadex A-50

The crude protein preparation was applied to an anion-exchange DEAE-Sephadex A-50 column (4 cm i. d. ×16 cm, Amersham Pharmacia Biotech Co., Uppsala, Sweden). Protein was eluted with a linear gradient of 0-2.0 M NaCl in the buffer and collected in 3 ml fractions. Each fraction was assayed for catalase activity and those containing the highest activity were retained.

Step 5: Hydrophobic-interaction chromatography on Phenyl-Sepharose CL-4B

The fractions retained previously were then concentrated and dialyzed against 50 mM potassium-phosphate buffer (pH 7.0). The dialysate was applied to a Phenyl-Sepharose CL-4B column (1.5 cm i.d. ×17 cm, Amersham Pharmacia Biotech Co.). Protein was eluted with a linear gradient of 1 0 M $(NH_4)_2SO_4$ in the buffer. Fractions (each 3 ml) were collected and assayed as described above. The active fractions were then dialyzed and loaded on the second column of the same size as the first one. The coloumn was eluted with a linear gradient of 0.5-0 M $(NH_4)_2SO_4$ in the buffer. One ml fractions were collected and assayed.

Molecular mass determination

The molecular mass of the native enzyme was determined by native PAGE using 7-20% gradient polyacrylamide gels (Atto Co., Tokyo). SDS-PAGE was performed with a 4.5% stacking gel and a 7.5% running gel to identify the sub molecular units. In both cases, the following standards (Amersham Pharmacia Biotech Co.) were used: bovine serum albumin (67.0 kDa), lactate dehydrogenase (140 kDa, tetramer), bovine liver

catalase (232 kDa, tetramer), ferritin (440 kDa, half unit; 220 kDa) and thyroglobulin (669 kDa, dimer). The gels were stained for protein using Coomassie Brilliant Blue.

Catalase activity staining

The following staining for catalase activity was done as reported previously (Clare *et al.*, 1984): the resulting native PAGE gel was soaked for 45 min in 50 mM potassium–phosphate buffer (pH 7.0) containing 50 μg/ml horseradish peroxidase (Wako Pure Chemical Industries Co., Osaka), followed by the addition of 5 mM H_2O_2 and the incubation at 25 °C for 10 min. The gel was washed twice with distilled water and then soaked in the buffer containing 0.5 mg/ml diaminobenzidine (Nacalai Tesque Co.) to develop the background brown color. No color would appear in the area where catalase depleted H_2O_2.

RESULTS

Purification of catalase

A single catalase was detected from the late-exponential and stationary growth phase cells of *S. warneri* ISK-1 by native PAGE and through catalase activity staining (data not shown). This result agrees with the previous report showing a single catalase from several *Staphylococcus* strains (Raymond, 1976). The enzyme was finally purified as described in Materials and Methods section. Twice operations of hydrophobic-interaction

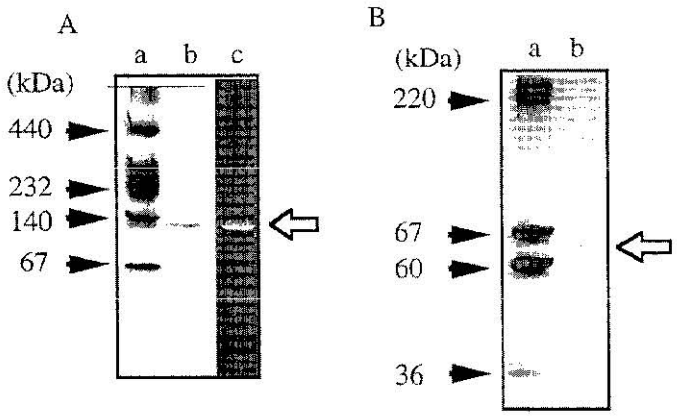

Fig. 1. Native and SDS-PAGE of catalase of *Staphylococcus warneri* ISK-1.
A, native PAGE with 7–20% gradient: lane a, protein-molecular-weight markers; lane b, native enzyme; lane c, catalase activity staining. The white arrow shows a native catalase. B, 7.5% SDS-PAGE: lane a, protein-molecular-weight markers; lane b, denatured enzyme. The white arrow shows a subunit of the catalase. The catalase was obtained from the culture broth of *Staphylococcus warneri* ISK-1 after 8 h cultivation (late-exponential growth phase) as described in Materials and Methods.

chromatography enhanced the purity of the isolated enzyme (Table 1). This resulted in a pure enzyme preparation as indicated by native and SDS-PAGE analysis (Fig. 1). The purification of the catalase as calculated by the final specific activity of 10800 U/mg was 310-fold with a 14% yield (Table 1).

Table 1. Purification of *Staphylococcus warneri* ISK-1 catalase

Step	Total volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Cell extraction[a]	69.0	1,590	55,000	35.0	100	1.00
Streptomycin sulfate precipitation	68.0	1,000	47,000	47.0	85.0	1.30
Ammonium sulfate precipitation	42.0	740	32,000	43.0	58.0	1.20
DEAE-Sephadex A-50	136	11.0	13,200	94.0	24.0	2.70
1st. Phenyl-Sepharose CL-4B	11.0	11.0	12,700	1,160	23.0	33.0
2nd. Phenyl-Sepharose CL-4B	9.00	0.70	7,600	10,800	14.0	310

[a] Cell extract was obtained from the culture broth of *Staphylococcus warneri* ISK-1 after 8 h cultivation (late-exponential growth phase) as described in Materials and Methods.

Absorption spectrum

The absorption spectrum of the purified catalase has been shown in Fig. 2. The absorption maximum at around 406 nm is characteristic of the protoheme IX group found in other monofunctional catalases (Claiborne *et al.*, 1979; Loprasert *et al.*, 1988; Walker *et al.*, 1995; Yumoto *et al.*, 1990).

Quaternary structure

The subunit Mr of the purified enzyme, determined under denaturing conditions was 64,000 (Fig. 1B). The Mr of the catalase band was estimated to be 125,000. These data indicated that the enzyme is dimeric, which is different from the tetrameric catalases. On the other hand, dimeric catalases so far purified have been found from the following bacteria: *S. venezuelae* (Knoch *et al.*, 1989), *Comamonas compransoris* (Nies and Schlegel, 1982), *Klebsiella pneumoniae* (Goldberg and Hochman, 1989a), *Mycobacterium tuberculosis* (Gruft and Gaafar, 1974) and *Bacteroides fragilis* (Rocha and Smith, 1995).

Relationship between cell growth and catalase activity

Figure 3 shows the relationship between cell growth and catalase activity. The results demonstrated that while no significant increase in the activity was detected throughout microaerobic condition, the activity at aerobic condition increased rapidly at late-exponential growth phase and decreased slowly on entering stationary growth phase. The increase in the specific activity with aeration was about three-fold as compared to that of microaerobic culture. As shown in Fig. 1A, no other catalases were specifically expressed by aeration. These indicate that the expression of single catalase of *S.*

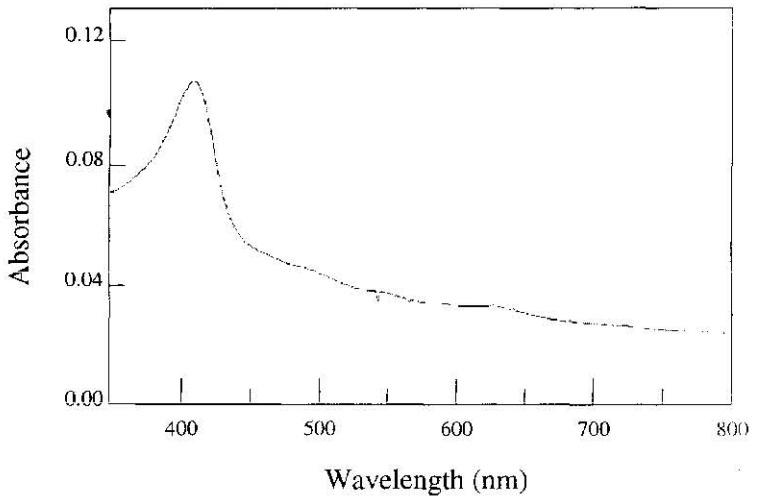

Fig. 2. Absorption spectrum of purified catalase of *Staphylococcus warneri* ISK-1.
Enzyme was dissolved in 50 mM potassium phosphate buffer (pH 7.0). The spectrum was recorded with a blank of 50 mM potassium phosphate buffer (pH 7.0).

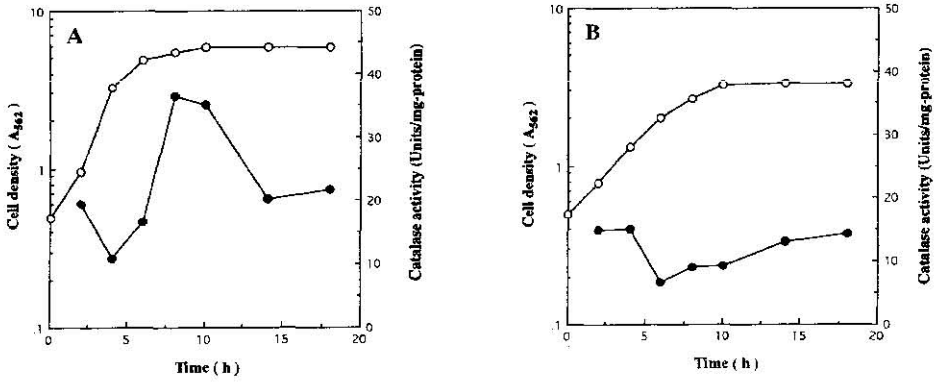

Fig. 3. Growth cycle and specific activity of catalase from *Staphylococcus warneri* ISK-1.
Cultures were grown at 37 °C in 1-l jar fermentor containing 500 ml MRS medium. A, aerobic condition: The air flow was carried out at a rate of 200 ml/min. B, microaerobic condition. ●, catalase specific activity. ○, cell density. The activity was measured three times independently and showed the average.

warneri ISK-1 was positively regulated by oxygen or the intermediates during aerobic respiration. Similar oxygen regulations of catalase gene expression have been reported in *Bacteroides fragilis* (Rocha and Smith, 1997) and *Lactobacillus sakei* LTH 677 (Hertel *et al.*, 1998).

Inhibitors

The effect of KCN, NaN_3 and 3-amino-1, 2, 4-triazol on the catalase activity was examined for a period of 30 min in each case. KCN (10 mM) and NaN_3 (10 mM) inhibited the catalase activity by 70 and 100%, respectively. These inhibitions are known to block the heme, activity center of catalases. Treatment with 500 mM of 3-amino-1, 2, 4-triazol which specifically inhibits monofunctional catalase also reduced the catalase activity by 87%.

Protein stability

The effects of ethanol/chloroform, pH and temperature were investigated. The purified catalase retained 100% of its activity when exposed to a mixture of ethanol/chloroform (enzyme solution: 95% ethanol: chloroform=10:5:3, by vol.) for 30 min with intermittent vortexing at room temperature. Catalase activity showed a broad maximum in the range of pH 5.5 to 9.5. It retained a maximum activity after heat treatment up to 40°C for 1 h. At 50°C, however, about 50% of the activity was lost, and the enzyme was completely inactivated at 60°C.

DISCUSSION

Staphylococcus is one of the most halotolerant eubacteria. *Staphylococcus aureus* can grow under high osmotic stress that occurs in medium containing up to 3.5 M NaCl (Scott, 1953). In addition, the osmoregulation of *S. aureus* was associated with the response to oxidative stress (Buisseret *et al.*, 1995). The property of halotolerance may make the response to oxidative stress distinctive from that of other bacteria like *E. coli*. We characterized a catalase of *Staphylococcus warneri* ISK-1 isolated from well aged *Nukadoko*, which contained a high NaCl concentration to prevent it from rotting. Therefore, it might possess a sophisticated adaptation mechanisms to oxidative stress as well as NaCl stress. *S. warneri* ISK-1 has a single catalase detected by catalase activity staining of native PAGE, which might indicate the possible relationship between the singleness of catalase and the character of its host described by Klotz *et al.* (Klotz *et al.*, 1997). Bacteria possessing only a single catalase have a restricted environment in parasitic life (*Helicobacter pyroli* (Odenbreit *et al.*, 1996), *Brucella abortus* (Sha *et al.*, 1994), *Compylobacter jejuni* (Grant and Park, 1995), *Bordetella pertussis* (Deshazer *et al.*, 1994), *Proteus mirabilis* (Jouve *et al.*, 1983), *Haemophilus influenzae* (Fleischmann *et al.*, 1995) and *Nesseria gonorrhoeae* (Archibald and Duong, 1986)). In addition, these catalases have a mutual sequence and form a distinct group within 74 catalase protein sequences from bacteria, fungal, animal and plant sources (Klotz *et al.*, 1997). The physiological significance of the singleness of catalase has been, however, not known yet.

The catalase from *S. warneri* ISK-1 shared general molecular properties with typical

monofunctional catalases in protein sensitivity towards temperature, pH and their specific inhibitors. The catalase did not exhibit peroxidase activity with diaminobenzidine, o-dianisidine and pyrogallol as expected (data not shown). However its regulation of expression is different from that of other bacteria. It is noteworthy that catalase activity of the cells cultured under aerobic condition decreased in the stationary growth phase. On the other hand, many studies have reported an apparent increase in catalase activity upon entry into stationary growth phase. In *E. coli katE* encoding HP II monofunctional catalase is expressed specifically in stationary growth phase and the catalase activity increases from 2 to 3 fold than in the exponential growth phase (Loewen *et al.*, 1985). This increase in activity during stationary growth phase was also found in *Streptomyces coelicolor* A3(2) although it has a single catalase (Walker *et al.*, 1995). Therefore, it is not only due to another specific catalase gene expression in stationary growth phase. Similar increases in catalase activity in stationary growth phase have been described in *Bacillus subtilis* (Lowen and Switala, 1987), *Pseudomonas fluorescens* (Rodriguez-Bravo and Pionetti, 1981), *Rhodopseudomonas spheroides* (Clayton, 1960), *Pseudomonas syringae* (Klotz and Hutcheson, 1992) and *Staphylococcus aureus* (Andrews and Martin, 1979). Thus, the role of catalase of *S. warneri* ISK-1 in the adaptation to stressful condition of stationary growth phase might not be crucial, so another adaptation response to oxidative stress like peroxidase, SOD or glutathione reductase might become more important. It would be interesting to measure the intracellular concentration of H_2O_2 and the activities of the other defence enzymes mentioned above in stationary growth phase.

We are currently investigating the regulation of catalase expression of *S. warneri* ISK-1 to establish its regulatory mechanism in adaptation to various stress conditions.

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