Journal of Biochemistry and Molecular Biology, Vol. 34, No. 3, May 2001, pp. 278-283

JOURNAL OF
Biochemistry and Molecular Biology

Short communication

Growth-Dependent Variations in Antioxidant and Redox Enzyme Activities of *Schizosaccharomyces pombe*

Young-Wook Cho, Eun-Hee Park[†], Ki-Sup Ahn[‡], Daemyung Kim[§] and Chang-Jin Lim*

Division of Life Sciences, Kangwon National University, Chuncheon 200-701,
[†]College of Pharmacy, Sookmyung Womens University, Seoul 140-742,
[‡]Department of Environmental Management, Chonan College of Foreign Studies, Chonan 330-180,
[§]Department of Genetic Engineering, Chongju University, Chongju 360-764, Korea

Received 29 December 2000, Accepted 21 February 2001

Antioxidant and redox enzyme activities are known to be involved in the cellular responses to various stresses. Their variations were observed according to the growth cycle of the fission yeast *Schizosaccharomyces pombe*. Peroxidase activity appeared to be notably higher in the early exponential phase than in the mid-exponential and stationary phases. However, catalase activity showed a variation pattern resembling the growth curve. Glutathione S-transferase activity was higher in the early exponential and late stationary phases. Activities of the two redox enzymes, thioredoxin and thioltransferase (glutaredoxin), were high in the stationary phase. However, their activities appeared to increase from the early exponential to mid-exponential phase. Total glutathione content had a varying pattern similar to that of thioredoxin and thioltransferase. However, its content in the early exponential phase was high. These results propose that antioxidant and redox enzymes tested are also involved in the mechanism of cell growth.

Keywords: Catalase, Fission yeast, Glutathione, Glutathione S-transferase, Peroxidase, *Schizosaccharomyces pombe*, Thioltransferase, Thioredoxin

Introduction

Elucidation of the cellular responses to metabolic stresses are needed, not only to understand basic cellular mechanisms, but also to maintain metabolic viability and the vitality of microbial strains used in industrial fermentations. Common stresses include: pH shock, temperature shock, oxidative stress, osmotic shock, and fermentation products toxicity. Oxidative stress is an unavoidable consequence of oxygen metabolism, and occurs in aerobic cells. The reactive oxygen species (ROS), such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH\cdot$), are produced by both normal aerobic metabolism and environmental agents. They can damage intracellular components, such as DNA, proteins, and membrane lipids (Ross *et al.*, 2000; Smirnova *et al.*, 2000). Consequently, they can result in mutagenesis, and inhibition of growth and cell death (Smirnova *et al.*, 2000), and are further implicated in aging, all stages of cancer, and numerous diseases (Halliwell, 1987).

Activities and protein levels of antioxidant enzymes, such as superoxide dismutase, peroxidase, catalase, and glutathione S-transferase, are closely linked with cellular responses to various oxidative stresses. Catalase is a very efficient enzyme that protects cells from the accumulation of H_2O_2 catalyzing the conversion of H_2O_2 to H_2O and O_2, and it is effective at high levels of H_2O_2 (Michiels *et al.*, 1994). When the natural antioxidant systems of *Caenorhabditis elegans* were augmented with small synthetic catalase mimetics, its life-span was significantly increased (Melov *et al.*, 2000). Catalase and thioredoxin peroxidase were found to act together to maintain cell viability, and protect *S. cerevisiae* mitochondria against Ca^{2+}-promoted membrane permeabilization (Kowaltowski *et al.*, 2000).

The term peroxidase (POD) in its widest sense includes a group of specific enzymes, such as NAD-POD, NADP-POD, fatty acid-POD, cytochrome-POD and glutathione-POD, as well as a group of very non-specific enzymes from different sources, which are simply known as POD (donor: H_2O_2-oxidoreductase, EC 1.11.1.7). POD catalyses the dehydrogenation of a large number of organic compounds, such as phenols and aromatic amines, hydroquinones and hydroquinoid amines, especially benzidine derivatives. With few exceptions, the typical POD belonging to group 1.11.1.7 are haemoproteins. Although the real POD reaction consists

*To whom correspondence should be addressed.
Tel: 82-33-250-8514; Fax: 82-33-242-0459
E-mail: cjlim@cc.kangwon.ac.kr

of the transfer of hydrogen from a donor to H_2O_2, there are examples of POD acting like oxidases ($SH_2 + O_2 \rightarrow S + H_2O_2$) and mono-oxygenases ($S\text{-}H + O_2 + NADPH \rightarrow S\text{-}OH + NADP^+ + OH^-$) (Pütter, 1974).

Glutathione S-transferase (GST; EC 2.5.1.18) catalyzes the addition of glutathione moiety to a great variety of acceptor molecules. Acceptors include carcinogens, organic hydroperoxides, and lipid peroxides, which cause oxidative stress in the cell (Homma and Listowsky, 1985). The conjugation is usually considered to be a detoxication, and the conjugate is degraded by the enzyme of the γ-glutamyl cycle. GST plays an important role in the protection mechanism against endogenous oxidative stress. GST also catalyzes essential steps in the biosynthesis of prostaglandins and leukotrienes (Angelucci *et al.*, 2000).

Redox regulation plays an important role at diverse levels of cellular functions, including the stress response. Thioredoxin (TRX) and thioltransferase (TTase) (also known as glutaredoxin) are small disulfide reducing enzymes, which have conserved consensus sequences -CXXC- at their active sites (Holmgren, 1989). Thioredoxin is a potent protein disulfide oxidoreductase, which is important in antioxidance defense (Nakamura *et al.*, 1997), regulation of cellular proliferation (Powis *et al.*, 1994) and the regulation of gene expression through transcription factor activation (Hayashi *et al.*, 1993). In addition, it is a powerful singlet oxygen quencher and hydroxyl radical scavenger (Das and Das, 2000).

TTase is a small and ubiquitous glutathione-dependent disulfide oxidoreductase. TTase participates in a pathway that couples the oxidation of NADPH to the reduction of ribonucleotide, sulfate, methionine sulfoxide, and arsenate (Holmgren, 1989). Some TTases are able to reduce non-disulfide substrates, such as dehydroascorbate (Wells *et al.*, 1990) and alloxan (Washburn and Wells, 1997). The OxyR transcription factor, which is activated through the formation of a disulfide bond, is deactivated by TTase (Zheng *et al.*, 1998). TTase may be involved in the regulation and maintenance of protease activity in HIV-1 infected cells (Davis *et al.*, 1997). The three-dimensional structure of a mammalian TTase contains an α/β structure with a four-stranded mixed β-sheet in the corn, flanked on either side by helices (Katti *et al.*, 1995).

Glutathione is the most abundant and ubiquitous nonprotein thiol in eukaryotic and prokaryotic cells. It plays an important role in protecting cells against oxidative damage, toxic compounds, radiation and heavy metals (Meister, 1983). Glutathione per se can react with oxidative agents, or is involved in the oxidative stress response through glutathione peroxidase (Penninckx, 2000). It has been reported that glutathione derived from glutamine promotes an inhibition of glutathione transport into mitochondria, which may render tumor cells more susceptible to oxidative stress-induced mediators (Carretero *et al.*, 2000).

This investigation was carried out with the assumption that antioxidant and redox enzymes might be involved in microbial cell growth. As expected, their activities were found to vary significantly, depending on the growth phase of *Schizosaccharomyces pombe* cells.

Materials and Methods

Chemicals Bovine serum albumin (BSA), yeast glutathione reductase (GR), NADPH, 1-chloro-2,4-dinitrobenzene (CDNB), Tris, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), oxidized glutathione (GSSG), leucine, uracil, adenine, guaiacol, and Bradford reagent were purchased from the Sigma Chemical Co. (St. Louis, USA). The 2-Hydroxyethyl disulfide (HED) was from the Aldrich Chemical Co. (Milwaukee, USA). All other chemicals and reagents used were of highest grade commercially available.

Cell growth and medium Wild-type *Schizosaccharomyces pombe* KP6 was cultured in YEALU, which contained 5 g yeast extract, 30 g glucose, 75 mg adenine, 75 mg leucine and 75 mg uracil in 1 L. Adenine, leucine and uracil were added for good growth of the yeast cells. The 2-day-grown culture was diluted 500-fold for inoculation. The yeast cells were grown at 30°C with shaking, and the cell growth was monitored by absorbance at the wavelength of 600 nm (Cho *et al.*, 2000a).

Cell harvest and disruption According to the time-course of shaking, the yeast cells were harvested by centrifugation. They were frozen and resuspended in a 20 mM Tris buffer (pH 8.0) -2 mM EDTA (buffer A), and disrupted by using a glass bead beater and a sonicator. Supernatant was obtained after centrifugation, and used as a crude extract for enzyme assays.

Peroxidase assay Peroxidase activity was determined using guaiacol as a hydrogen donor, according to the published procedure (Pütter, 1974). The reaction mixture (1 ml) contained a 0.1 M phosphate buffer (pH 7.0), 0.316 mM guaiacol, 0.116 mM hydrogen peroxide, and the appropriate volume of the crude extract. The change in absorbance was measured at 436 nm. Specific activities of peroxidase were indicated as A_{436}/min/mg protein.

Catalase assay Catalase activity was determined by monitoring the decrease in absorbance at 240 nm due to H_2O_2 consumption (Rao *et al.*, 1996; Cho *et al.*, 2000b). The 3 ml reaction mixture contained a 100 mM phosphate buffer (pH 7.0) and 10 mM H_2O_2. The mixture was incubated for 2.5 minutes at 30°C. The reaction was initiated by adding the crude extract. The decrease in absorbance was then recorded for a few minutes. Specific activities of catalase were indicated as A_{240}/min/mg protein.

Thioredoxin assay Oxidized thioredoxin was tested as a substrate of *E. coli* thioredoxin reductase in the presence of NADPH and DTNB (Luthman and Holmgren, 1982; Lee *et al.*, 2000). In a final volume of 0.5 ml, the reaction mixture contained a 100 mM Tris-HCl buffer (pH 8.0), 2 mM EDTA, 0.5 mM DTNB (dissolved in 95% ethanol), 0.24 mM NADPH, and crude extract. The reaction was started by adding *E. coli* thioredoxin reductase, and the reduction of DTNB was determined by monitoring the

absorbance change at 412 nm. The specific activity was expressed as A_{412}/min/mg protein.

Thioltransferase assay Thioltransferase catalyzes the reduction of certain disulfides in the presence of glutathione, and thus has GSH-disulfide-transhydrogenase. In the present study, 2-hydroxyethyl disulfide was used as a substrate (Cho *et al.*, 1999; Kim *et al.*, 1999a; Park *et al.*, 1999; Cho *et al.*, 2000c). Two quartz semimicro cuvettes with a 10-mm light path contained 500 μl of mixture at room temperature. Both cuvettes contained 100 μg/ml of bovine serum albumin, 1 mM GSH, 6 μg/ml yeast glutathione reductase, 0.4 mM NADPH, and 0.1 M Tris-HCl (pH 8.0) -2 mM EDTA. The absorbance at 340 nm was recorded for 2 minutes to ensure that both cuvettes were balanced with respect to the nonenzymatic spontaneous reaction between glutathione and 2-hydroxyethyl disulfide. The cell extract was added to the sample cuvette, and an equal volume of buffer A was added to the reference cuvette. The decrease in absorbance was then recorded for a few minutes. The result was calculated as A_{340}/min/mg protein.

Glutathione S-transferase assay Glutathione S-transferase (GST) activity was spectrophotometrically determined as previously described (Habig *et al.*, 1974; Kim *et al.*, 1999b; Cho *et al.*, 2000d) with minor modifications. The reaction mixture contained a 100 mM phosphate buffer (pH 6.5), 5.0 mM GSH, 2.0 mM CDNB, and a crude extract in a volume of 1.0 mL. The reaction, conducted at 25°C, was initiated by the addition of CDNB, and the change in the absorbance at 340 nm was monitored with a spectrophotometer. All initial rates were corrected for the background nonenzymatic reaction. Specific activities of glutathione S-transferase were calculated as A_{340}/min/mg protein.

Total GSH Total GSH content was determined by the absorbance at 412 nm (Sies and Akerboom, 1976) using oxidized GSH (GSSG) as a standard. In a final volume of 0.5 ml, the reaction mixture contained a 100 mM phosphate buffer (pH 7.0) -1 mM EDTA, 0.24 mM NADPH, 0.0756 mM DTNB, and 0.06 units GR. Then 100 μl of the appropriate GSSG standard, or 100 μl of the crude extract, was added to each of the cuvettes. The absorbances, obtained from known concentrations of GSSG, were used to construct a standard curve. Total GSH content was calculated as ng/mg protein.

Protein determination Protein concentration was determined according to the procedure of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Results and Discussion

When the aerobic cells grow, reactive oxygen species and other harmful compounds may be produced from their normal metabolic processes, and inhibit cell growth. Defense enzyme systems would be required for the normal growth of the cells. In this investigation, the fission yeast *S. pombe* cells were grown in a rich medium, and antioxidant and redox enzyme activities were analyzed according to the growth cycle.

Antioxidant enzymes *S. pombe* peroxidase activity appears to be significantly high at the early exponential phase (Fig. 1). However, when the yeast cells enter the mid-exponential phase, it is greatly decreased, and remains at approximately 20% of the early exponential phase activity (Fig. 1). Even in the stationary phase, the low activity of peroxidase is

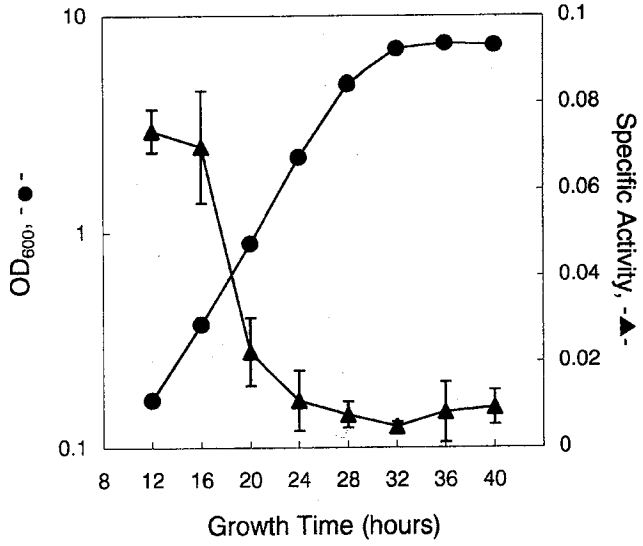

Fig. 1. Growth-dependent variation in peroxidase activity of wild-type *S. pombe* cells. The yeast cells were grown in a YEALU medium, and harvested at the indicated time intervals. Peroxidase activity (-▲-) was determined as described in 'Materials and Methods', according to the growth curve (-●-). Specific activities of peroxidase were represented by A_{436}/min/mg protein. Each value indicates a mean obtained from the three experiments.

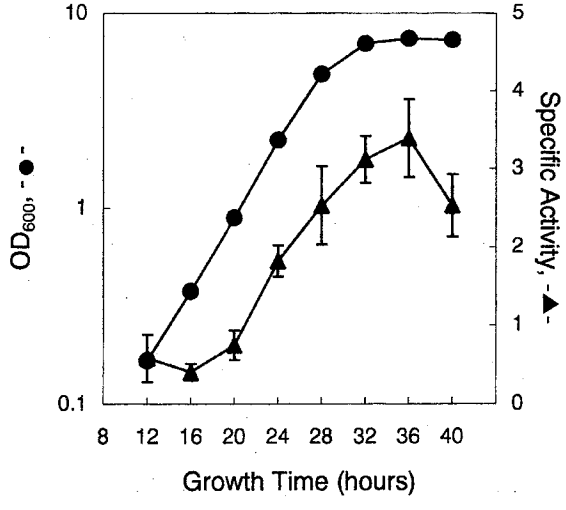

Fig. 2. Growth-dependent variation in catalase activity of wild-type *S. pombe* cells. The yeast cells were grown in a YEALU medium, and harvested at the indicated time intervals. Catalase activity (-▲-) was determined as described in 'Materials and Methods', according to the growth curve (-●-). Specific activities of catalase were represented by A_{240}/min/mg protein. Each value indicates a mean obtained from the three experiments.

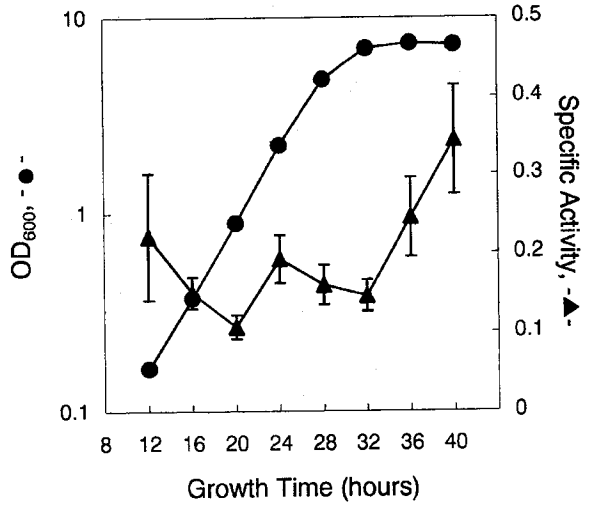

Fig. 3. Growth-dependent variation in glutathione S-transferase activity of wild-type *S. pombe* cells. The yeast cells were grown in a YEALU medium, and harvested at the indicated time intervals. Glutathione S-transferase activity (-▲-) was determined as described in 'Materials and Methods', according to the growth curve (-●-). Specific activities of glutathione S-transferase were represented by A_{340}/min/mg protein. Each value indicates a mean obtained from the three experiments.

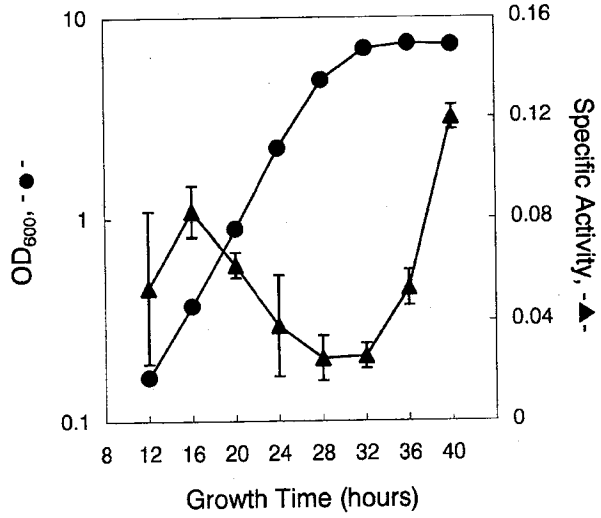

Fig. 4. Growth-dependent variation in thioredoxin activity of wild-type *S. pombe* cells. The yeast cells were grown in a YEALU medium, and harvested at the indicated time intervals. Thioredoxin activity (-▲-) was determined as described in 'Materials and Methods', according to the growth curve (-●-). Specific activities of thioredoxin were represented by A_{412}/min/mg protein. Each value indicates a mean obtained from the three experiments.

preserved. It is elevated in the long-term culture of *S. pombe* cells. These results propose that peroxidase may play more important roles in the newly dividing *S. pombe* cells. Catalase activity, catalyzing the conversion of H_2O_2 to H_2O and O_2, is notably increased during the exponential phase (Fig. 2). At the early stationary phase, catalase activity goes up to 6-fold of the early exponential phase activity. Its varying pattern shows a shape resembling the growth curve. However, at the later stationary phase, catalase activity begins to drop. Catalase activity seems to have an active function in the dividing *S. pombe* cells. The physiological meaning of these findings remains elusive. In the peroxidase assay, guaiacol was used as a hydrogen donor. Varying patterns in other kinds of peroxidase would be very interesting. The *S. pombe* glutathione peroxidase gene was found to be induced by various stresses, including oxidative stress, osmostress and heat stress (Yamada *et al.*, 1999). These stresses activate the Wis1-Sty1/Spc1 MAP kinase cascade in *S. pombe*. The transcriptional factor Atf1 and Pap1 are under the control of this MAP kinase. A transcriptional factor, Atf1, and its binding site, are also necessary for the induction of the *S. pombe* catalase gene by osmotic stress, UV irradiation, and heat shock (Nakagawa *et al.*, 2000). However, the factor responsible for the induction of the catalase gene by oxidative stress was identified as the transcription factor Pap1. However, the relationship between these identified regulations, and the growth phase-dependent variations, is unknown. Understanding the growth-dependent regulation of peroxidase and catalase may expand their physiological roles. Glutathione S-transferase activity was found to be elevated in

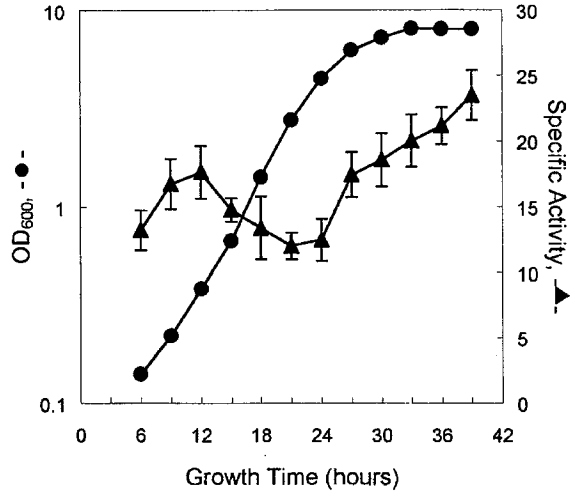

Fig. 5. Growth-dependent variation in thioltransferase activity of wild-type *S. pombe* cells. The yeast cells were grown in a YEALU medium, and harvested at the indicated time intervals. Thioltransferase activity (-▲-) was determined as described in 'Materials and Methods', according to the growth curve (-●-). Specific activities of thioltransferase were represented by A_{340}/min/mg protein. Each value indicates a mean obtained from the three experiments.

both the early exponential and the late stationary phases (Fig. 3). The mid-exponential GST activity appeared to be relatively low. GST activity may have more significant functions in non-dividing *S. pombe* cells. There are at least three GST genes in *S. pombe* genomic DNA, estimated from

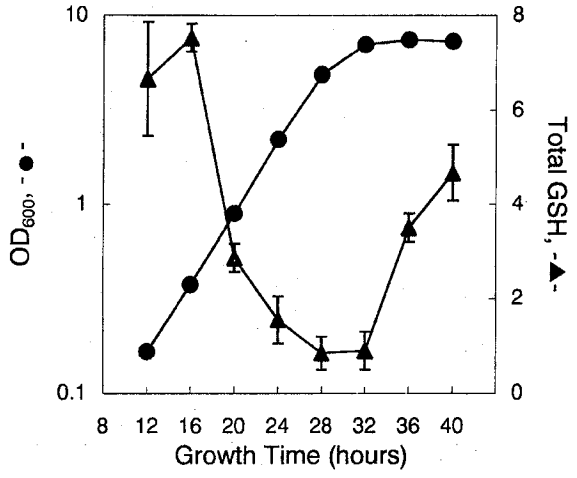

Fig. 6. Growth-dependent variation in glutathione content of wild-type *S. pombe* cells. The yeast cells were grown in a YEALU medium, and harvested at the indicated time intervals. Total glutathione content (-▲-) was measured as described in 'Materials and Methods', according to the growth curve (-●-). Total glutathione content was represented by ng/mg protein. Each value indicates a mean obtained from the three experiments.

GenBank database (data not shown). One of them was isolated, and its induction occurred in the lag phase *S. pombe* cells (data not shown). The growth-dependent variation in *S. pombe* GST activity can be explained on the genetic level through extensive investigation. The function of *S. pombe* GST activity may be related to the viability of the non-dividing cells.

Redox enzymes Two redox enzymes, thioredoxin and thioltransferase, are believed to be involved in cellular responses to oxidative stresses. Thioredoxin activity is extensively required for rapidly growing mammalian cells (Grogan *et al.*, 2000). The two enzyme activities were compared according to the growth cycle of *S. pombe* cells. As shown in Fig. 4, thioredoxin activity appears to be higher in both the early exponential and stationary phases than in the mid-exponential phase. In case of thioltransferase, the similar variation pattern can be obtained (Fig. 5). Thioltransferase activity varies in a relatively narrow range. High activities of thioredoxin and thioltransferase in non-dividing *S. pombe* cells was unexpected. They might be needed to preserve a suitable redox status in the non-dividing cells. However, *E. coli* thioredoxin is induced at the stationary phase. One *S. pombe* thioltransferase gene was cloned, and its expression was enhanced by zinc and NO-generating S-nitroso-N-acetylpenicillamine (Cho *et al.*, 2000c). Growth-dependent regulatory mechanisms of *S. pombe* thioredoxin and thioltransferase genes remains to be solved.

Total GSH GSH is present in a high concentration in most living cells from microorganisms to humans, and serve as an endogenous sulphur source in the yeasts. GSH is known to be involved in the responses to various stresses (Penninckx, 2000). However, GSH has received comparatively little attention and has often been ignored. GSH content was measured during the growth cycle of *S. pombe* cells. It appears to be significantly higher in the early exponential and stationary phases than in the mid-exponential phase. Unexpectedly, it was the lowest in the actively dividing phase. This might indicate that GSH also plays a physiological role in the survival of non-dividing cells.

In this communication, we observed that the activities of the three antioxidant enzymes, and two redox enzymes, varied according to the growth cycle of the fission yeast. Although they are known to be concerned in the stress response, our results indicate their participation in the cellular growth. However, their action mechanism remains to be elucidated. Their growth-dependent regulatory mechanisms would be interesting projects.

Acknowledgments This work was supported by a grant (No. 2000-1-20900-004-3) from the Basic Research Program of the Korea Science & Engineering Foundation.

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