JSc-EUSL(2000) **Vol.1 No.1**, p 40-51

ISSN 1391-586X; ©2000 Published by Faculty of Science, Eastern University, Sri Lanka

GLUTATHIONE IS AN ESSENTIAL METABOLITE REQUIRED FOR RESISTANCE TO OXIDATIVE STRESS AND INDUCTION OF STRESS TOLERANCE IN YEAST *S*accharomyces cerevisiae

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(Received 26 June 2000; accepted 1 March 2001)

Abstract

Yeast cells were depleted of glutathione by treatment with iodoacetamide and were markedly more sensitive to damage caused by free radicals generated by AAPH (2,2 '-azobis(2-amidinopropane) dihydrochloride) and H_2O_2. However intrinsic tolerance to heat and ethanol were not affected. Glutathione depletion inhibited the induction of tolerance to all four stresses and the induction of heat shock protein (hsp) synthesis. It was concluded that glutathione was essential for oxidative stress tolerance and induction of tolerance to oxidative, heat and ethanol stresses.

keywords: :Free radicals, Heat shock proteins, Glutathione, Yeast stress tolerance.

1 Introduction

The tripeptide glutathione (GSH) is one of the most important non-enzymatic defense molecule against oxidative stress, especially caused by peroxide generating agents in many organisms including yeast [1][2][3]. Glutathione peroxidase and several other membrane bound enzymes such as glutathione transferase utilize glutathione as a co-factor during the detoxification process [4].

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The role of glutathione in hyperthermic injury in mammalian cells has been investigated for some time with conflicting results. Some reports failed to establish a relationship between heat sensitivity and constitutive levels of GSH [5] while others indicate a relationship between GSH content and heat shock induced thermotolerance [6]. However there are no such reports in the case of yeast.
Experiments using mutants defective in glutathione synthesis have shown that such cells are more sensitive to H_2O_2 [2][3] and superoxide anions [3].
Another means of elucidating the role of glutathione in stress tolerance is by depleting cells of GSH using chemicals including iodoacetamide which results in the depletion of glutathione by the formation of glutathione-s-conjugates. Such studies have also resulted in conflicting results in that some concluding that there was a correlation between the ability of various chemicals to deplete glutathione and their increased toxicity[7] while others reporting that there was no obvious correlation[8]. The conflicting reports on the role of glutathione in stress tolerance indicated that there were other factors involved in oxidative stress tolerance. It was decided to examine the role of heat shock proteins and glutathione using iodoacetamide. A number of other stresses were also examined in order to gain a wider picture. Free radicals were generated as peroxyl radicals produced by AAPH (2,2 ′-azobis(2-amidinopropane) dihydrochloride) and H_2O_2. Our study found that glutathione is an important antioxidant which protects cells against free radicals but not ethanol or heat stress.

2 Materials and methods

2.1 Yeast strains and culture conditions

Saccharomyces cerevisiae strain K7, a sake brewing yeast (ATCC 26422, American type culture collection, Rockville, USA), was used in the present studies. This strain has been previously used for studies on stress tolerance, including oxidative[9], heat[10] and ethanol[11] stresses. Cultures were grown at 25^0C in an orbital shaker at 200 *rpm* in YEPG media (0.5% yeast extract, 0.5% bacteriological peptone, 0.03% $(NH_4)_2SO_4$, 0.3% KH_2PO_4 and 2% glucose). Cells were harvested in exponential phase, corresponding to an optical density at 600 *nm* of between 0.2 and 0.4 and a cell density of around 5×10^6 *cells/ml*.
Preliminary experiments were carried with the glutathione depleting agent, iodoacetamide. Cells were treated with different concentrations ranging from 0.25 to 2 *mM* at 25^0C for upto 2 *hours*. The viability and glutathione contents were measured at different time intervals. The data indicated that treatment of cells with 1 *mM*-iodoacetamide for 30 *minutes* was optimum for depletion of glutathione for the purposes.

2.2 Determination of total glutathione content

The culture (10 *ml*) was centrifuged at 2500 *g* for 5 *minutes* and the cells washed several times in cold distilled water. The final pellet re-suspended in 10 *ml* of distilled water. Cell suspensions were boiled for 3-5 *minutes* and then rapidly chilled in an ice-bath. Suspensions were centrifuged and the supernatant transferred to clean tubes and placed on ice until assayed for GSH content. The total glutathione was estimated following the reduction of (oxidized form of glutathione) GSSG by 5,5 *I*-dithiobis(2nitrobenzoic acid) [DTNB] in the presence of glutathione reductase as previously described[12]. Assays were done in duplicate and glutathione concentrations obtained from a standard curve (Figure 5). Concentrations were expressed as $\mu moles/g$ dry weight. Each experiment was repeated a minimum of three times.

2.3 Dry weight

Dry weight measurements were determined by harvesting 80-100 *ml* of culture by centrifugation at 2500 *g* for 5 *minutes*. The cell pellet was washed twice in water to remove the growth medium and re-suspended in 10 *ml* of chilled water. The re-suspended cells were filtered onto pre-weighed Millipore filters (0.22 μm). Flters were dried for 24 *hours* at 80^0C. Filters were transferred into a dissector, allowed to cool and weighed.

2.4 Stress tolerance studies

The azo compound (2,2 *'*-azobis(2-amidinopropane) dihydrochloride) [Wako,Tokyo] and H_2O_2 were used to generate free radicals as peroxyl radicals. Cells from 20*ml* culture were suspended in 50 mM phosphate buffer (pH 5.7) and were treated with 1 mM iodoacetamide for 30 *minutes* at 25^0C in an oscillating water bath. Cells were then washed free of iodoacetamide by centrifuging at 2500*g* for 5 *minutes*, the pellet re-suspended in the same volume of the fresh phosphate buffer and were subjected to the following stresses 2,2 *'*-azobis(2-amidinopropane) dihydrochloride (15 mM AAPH at 42^0C), H_2O_2 (10 mM at 25^0C) or ethanol (17% v/v at 25^0C) over a 2 *hours* time course. For heat stress, iodoacetamide treated cells in phosphate buffer (50 mM, pH 5.7) were rapidly heated to 48^0C and incubated in an oscillating water-bath set at 48^0C. In heat shock experiments, cells were heated to 37^0C in an oscillating water-bath for 45 *minutes* prior to exposure to the above stresses over a 2 *hours* time-course. All studies were carried out in iodoacetamide treated as well as untreated cells. In all cases, cell viability was measured (in duplicate) by taking samples at appropriate intervals over the 2 *hours* time-course and plating onto YEPG agar. Colony forming units (cfus) were counted after incubation for 2 to 3 days at 25^0C. Percentage survivors were expressed as **cfu** after the stress relative to non-stressed sample. All the experiments were repeated at least three times.

2.5 Heat shock protein synthesis

Washed cells (40 *ml* culture) were re-suspended in 2 *ml* of YNB (0.67% yeast nitrogen base, 2% glucose) and incubated at the appropriate temperature (25^0C or 37^0C) for 5 *minutes* before addition of ^{35}S-methionine (10 μCi, specific activity 1150 $Ci\ mmol^{-1}$). Samples were incubated for 45 *minutes* and the reaction terminated by the addition of cold methionine (1 mM). Cells were harvested by centrifuging the suspension at 2500 g for 3 *minutes*, washed and proteins extracted as previously described[13]. Proteins were analyzed by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a running gel of 12% SDS. Equal amounts of (10 μg) of proteins were loaded into each well and samples run until the loading dye (bromophenol blue) reached the bottom edge of the gels. Gels were dried and autoradiographs developed using Biomax MR film (Kodak). Heat shock proteins (hsps) were designated by reference to protein molecular weight markers and specific antibodies were used to confirm the presence of hsps by Westernblot analysis previously described[13]. Antibodies used were hsp 104 and hsp 90 (Afinity bioreagents, New Jersey) and hsp 70 and hsp 60 (StressGen, Victoria, Canada).

3 Results

3.1 Glutathione concentrations

Glutathione concentrations were substantially reduced on treatment of cells with iodoacetamide as illustrated in Table. 1

Table 1: Glutathione content of K7 cells during different treatments [2]

Treatment	Glutathione content $\mu moles/g$ dry weight
25^oC (Control)	141.9 ± 3.9
Iodoacetamide at 25^oC(1 $mM, 30minutes$	$49.5 \pm 1.03.9$
Heat shock($37^oC, 45minutes$)	219.8 ± 15.2
Heat shock+ iodoacetamide ($1mM, 37^oC, 45minutes$)	81.9 ± 6.7
AAPH at 25^oc ($15mM, 45minutes$)	180.8 ± 9.9
AAPH at 42^oC ($15mM, 45minutes$)	96.6 ± 6.8

Assays were done in duplicate and experiments were performed at least three times. Values given are average of the three experiments with standard deviations.

Treatment with iodoacetamide at 25^0C for 30 *minutes* reduced glutathione contents to less than 35% of the control cells. On the other hand in cells subjected to a heat shock (37^0C, 30 *minutes*) in the presence of iodoacetamide glutathione levels were

reduced to about 58% indicating that there was increased synthesis of glutathione at this temperature. A heat shock alone at 37^0C for 45 *minutes* significantly increased glutathione levels. Exposure of cells to AAPH (15 mM) at 42^0C decreased levels to about 68%. Having established conditions where by the glutathione concentrations could be modulated, the effects of free radical and heat stress on cell viability was examined next.

3.2 Stress tolerance

It is known that the generation of free radicals by AAPH is temperature dependent [14]. It was important to establish conditions in which the effects of free radical stress, as generated by AAPH, are differentiated from the effects of temperature stress. In the present studies, we established that 42^0C was an optimum temperature. At this temperature, yeast cell viability was high (90% viable cells after 2 *hours*) while at 45^oC or higher cell viability markedly decreased over a 2 *hours* time course (20% viable). As illustrated in Figure 1, in all case, pretreatment of cells ith iodoacetamide sensitized cells to oxidative stress as compared to control, non-iodoacetamide treated cells (Figure 1 A and Figure 1 B). Iodoacetamide treated cells were 10-fold more sensitive to 10 mM- H_2O_2 and 15 mM AAPH intrinsically during the 2 *hours* time-course. However the viability of cells treated with iodoacetamide subjected to heat and ethanol stress were similar to that of the cells which were subjected to the above stresses without iodoacetamide treatment (Figure 1 C and Figure 1 D). This indicates that intrinsic tolerance to ethanol and heat were not greatly affected by iodoacetamide treatment. As expected , a mild heat shock (37^0C for 30 *minutes*) induced tolerance to all four stressors. On the other hand, heat shock in the presence of iodoacetamide rendered cells more sensitive than the intrinsic tolerance to all four stressors.

3.3 Iodoacetamide and protein profiles

Heat shock protein (hsp) synthesis was measured at 25^0C, $37^0C$with or without iodoacetamide as described in the methods. The protein profiles of cells at 25^0C, with or without iodoacetamide treatment were essentially the same except for bands observed at circa. 40 - 42 kDa and 47 kDa was probably hsp 47, since the same protein band was also induced upon a heat shock. Interestingly, the 40 kDa protein was also induced when cells were heat shocked in the presence of iodoacetamide (Figure 2).

Autoradiographs of SDS-PAGE were also processed for densitometric analysis and results for hsp 104, 90, 70, 60 and 47 are presented in Figure 3. Results are presented as fold-increase in synthesis at 37^0C relative to appropriate 25^0C control cell and using actin (43 kDa protein) as a standard for non heat shock inducible protein[13] to normalize pixel intensities on densitometric traces.

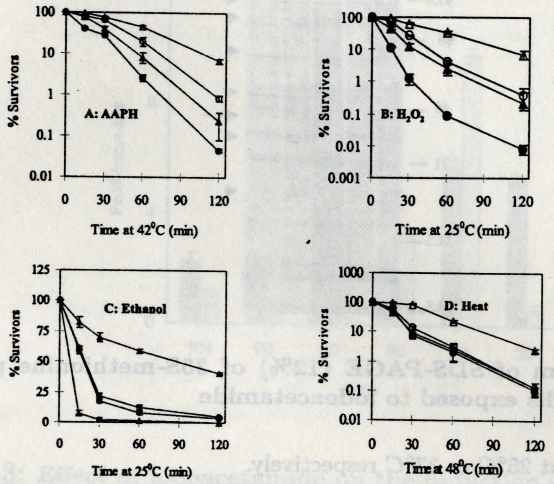

Figure 1: Effect of iodoacetamide on stress tolerance.

Cells were exposed to **(A)** AAPH (15 mM, 42°C), **(B)** H_2O_2 (10 mM, 25°C), **(C)** ethanol (17% v/v, 25°C) or **(D)** heat stress over a 2 hour time course.

(Open circle) Cells subjected to stress without a prior incubation with iodoaetamide Control

(Closed circle) Cells subjected to stress after incubation with iodoacetamide (1 mM, 25°C, 30 *minutes*)

In some experiments, cells were exposed to a heat shock (37°C for 45 min) prior to exposure to AAPH, H_2O_2 , ethanol or heat, with or without iodoacetamide

(Open triangle) Cells exposed to a heat shock (37°C for 45 *minutes*) without iodoacetamide prior to exposure to the stress (AAPH, H_2O_2, ethanol or heat)

(Closed triangle) Cells exposed to a heat shock (37°C for 45 *minutes*) with iodoacetamide prior to the exposure to the stress (AAPH, H_2O_2, ethanol or heat)

According to Figure 3 at 37^0C synthesis of hsp 104, 90,70 and 36 was significantly inhibited by iodoacetamide. In contrast treatment of cells with iodoacetamide at 37^0C appeared to induce the synthesis of proteins corresponding to hsp 60 and hsp 47. These results were also confirmed by Western immunoblot analysis with corresponding antibodies (Figure 4).

4 Discussion

Treatment of cells with iodoacetamide lead to a marked depletion of glutathione content. Cells thus treated were hypersensitive to oxidative stress. These observations on their own would suggest that glutathione is an important antioxidant

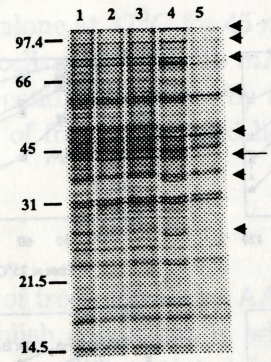

Figure 2: **Autoradiogram of SDS-PAGE (12%) of 35S-methionine protein extracts from control cells and cells exposed to iodoacetamide**

Lanes 1, 4 : Control cells at $25°C$ or $37°C$ respectively.
Lane 2, 5 : Cells exposed to iodoacetamide (1 mM,30 *minutes*) at 25 or $37°C$ respectively.
Lane 3 : Cells exposed to iodoacetamide (1 mM, 30 *minutes*) at $25°C$ and were washed free of iodoacetamide by centrifuging at 2500g for 5 *minutes*, the pellet re-suspended in the same volume of YNB and were incubated (left to recover) at $25°C$ for 45 *minutes*. Standards marked on the left. Large arrow indicates the protein induced by iodoacetamide at $25°C$ Arrows indicate, from the top, hsps,104,90,70,60,47,36 & 27; hsps were designated by reference to protein molecular weight markers and specific antibodies were used to confirm the presence of hsps by Westernblot analysis (results of Westernbolt analysis is illustrated in Figure.4).

defense system against oxidative stress. Enhanced susceptibility to metal chelating agent 8-hydroxyquinolone by GSH depletion in S. cerevisiae has been previously reported[8]. The present study has extended these observations to now include sensitivity to the free radical generator AAPH. On the other hand intrinsic tolerance to ethanol and heat were not affected. These results thus suggest that glutathione acts as a primary defense against oxidative stress and less so against ethanol or heat.
Glutathione levels also decreased by exposure of cells to AAPH at 42^0C, although not to levels as low as after iodoacetamide treatment. This could be due the detoxification of the radicals produced by AAPH by glutathione. Further support for this concept comes from the observation that cells heat shocked at 42^0Cin the absence of AAPH, showed substantial increase in the glutathione content (Table 1). This is a novel finding in yeast although heat shock induced synthesis of glutathione has been well documented in mammalian systems[15].
A mild heat shock (37^0C, 45 *minutes*) in the presence of iodoacetamide, blocked the classical observation of heat shock induced stress tolerance (Figure 1) and also the synthesis of hsps (Figure 2 and Figure 3). This observation could be interpreted as due to the depletion of glutathione in cells treated with iodoacetamide. However

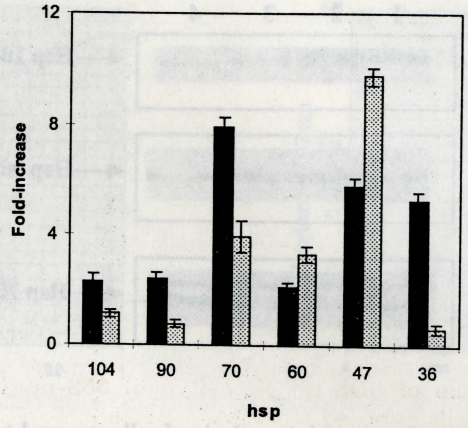

Figure 3: **Effect of iodoacetamide on the induction of hsp synthesis**

Cells were subjected to a $37°C$ heat shock for 45 *minutes*. Proteins were labelled and separated by SDS-PAGE. Bands corresponding to hsps were analysed by densitometer. Results expresed as fold-increase of protein bands of heat shocked cells in relation to control cells at $25°C$. Some cells were exposed to 1 mM iodoacetamide for 30 *minutes* at $25°C$ prior to a heat shock at $37°C$ for 45 *minutes*.Cells were heat shocked with [**filled dark black**] or without [**filled light gray**] prior iodoacetamde treatment.

it was not possible, at least under the present experimental conditions, to clearly differentiate the effects of glutathione and hsp on heat shock induced stress tolerance.

The present experiments, however did indicate the proteins corresponding to hsp 47 and hsp 60 were induced to higher level in cells heat shocked in the presence of iodoacetamide (Figure 2 and Figure 3). It is not immediately obvious why these proteins should be induced under these conditions. The known function of hsp 60 is a molecular chaperone, localized in the mitochondria. Depletion of glutathione could lead to increased oxidative damage to proteins and there by the amount of unfolded or missfolded proteins. This in turn would necessitate the need for increased chaperone activity. A requirement for an increase of hsp 47 (enolase) in glutathione depleted cells is unclear.

In addition to these two proteins, a protein at circa. 42 kDa was also induced in cells pretreated with iodoacetamide (Figure 2). The nature and function of this protein also remains to be determined. Further studies using different sulfhydryl compounds such as buthionine sulfoxime may prove informative in this respect.

As far as one is aware, there are no published data on glutathione depletion associated with stress protein synthesis in yeast. In this respect, the present observations

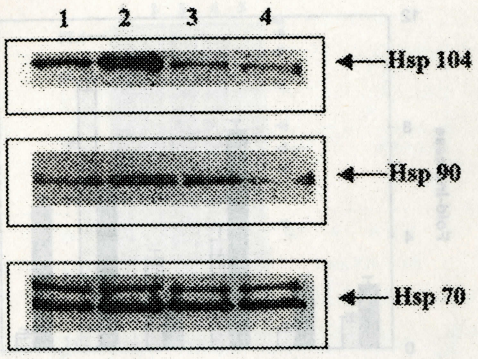

Figure 4: Western immunoblot analysis of cells exposed to iodoacetamide

Cells were exposed to 1 mM iodoacetamide at 25°C for 30 *minutes* and then heat shocked at 37°C for 45 *minutes*

Lanes 1, 2: Control cells at 25°C and cells exposed to a 37°C heat shock for 45 *minutes* respectively.

Lane 3: Cells exposed to iodoacetamide at 25°C for 30 *minutes*

Lane 4: Cells exposed to idoacetamide at 25°C for 30 *minutes* and heat shocked at 37°C for 45 *minutes*

are novel. There is however, a few recent reports on stress protein synthesis and glutathione levels in mammalian cells. In rat glioma cells, depletion of glutathione levels by diethylmalate or buthionine sulfoxime, lead to increased expression of hsps 27, 70 and 90 [15]. The mechanism for this observation was unclear, however, there was some evidence that altered binding of heat shock factor (HSF1) to heat shock element (HSF) may be involved. In another study, using cardiac myocyte cells, increased glutathione content induced by ebelsen, a seleno-organic compound, lead to increased synthesis of hsp 70 and heme oxygenase[16]. Interestingly, it was reported that eblesen pretreatment resulted in reduced damage to cells by H_2O_2.

These studies, taken in conjunction with the present observations, support the proposal of a relationship between glutathione metabolism and stress protein synthesis. It may be anticipated that elucidation of the mechanism of this interaction would make a substantial contribution towards our understanding of the role of glutathione in yeast stress tolerance.

In summary, we have shown that glutathione is an effective antioxidant against free radical and oxidative stress and not against ethanol or heat stress. In addition a correlation between heat shock induced tolerance and heat shock protein synthesis with glutathione content could also be suggested although further studies would be

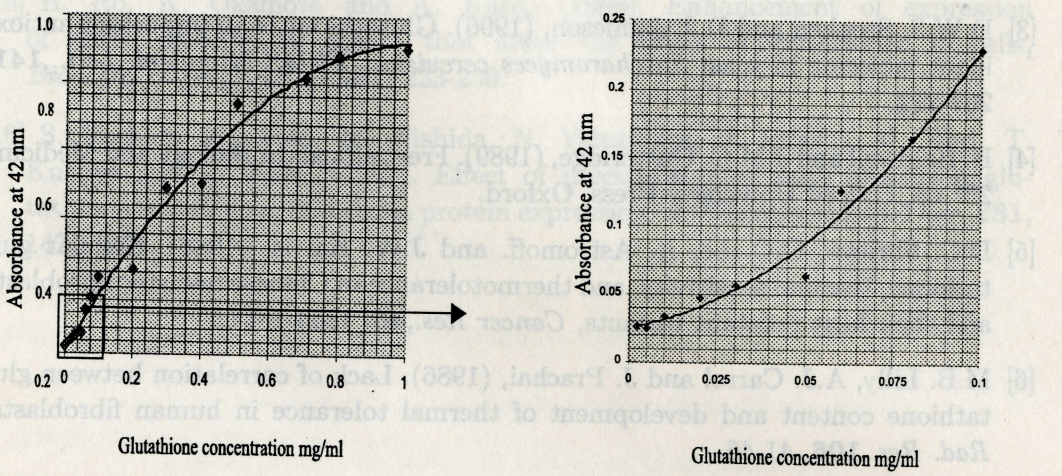

Figure 5: **Standard curve for glutathione concentrations A- Glutathione concentrations from 0 -1** *mg/ml* **B-Glutathione concentrations from 0.01- 0.1** *mg/ml*

A series of different concentrations of yeast glutathione (baker's yeast , Sigma) was prepared. 1 *ml* of Sample was added to the assay mixture containing the following : 1 *ml* of buffer [0.5 *mM* potassium phosphate buffer (pH 7.1)-1 *mM*-EDTA (pH 7.5) 80:45], 0.04 *ml* of DTNB solution [40 *mg* of DTNB in 10 *ml* of 1.5 *mg/ml* $NaHCO_3$ in 0.1 *mM* potassium phosphate buffer (pH 7.1)], 0.02 *ml* of NADPH solution (4 *mg* NADPH in 1 *ml* of 0.02 N-NaOH) and 0.002 *ml* of bakers yeast glutathione reductase (Sigma type III in 3.6 *M* $(NH_4)_2SO_4$ solution pH 7.0). Mixture was incubated at room temperature and the absorbance was measured at 412 *nm* after 3 *minutes*. Assays were done at least three times and the values plotted are the average.

needed to confirm the above.

Acknowledgements

This work was supported by a scholarship from AUSAID and internal research grants from The University of New England.

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