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RESEARCH ARTICLE

Protein expression profiles in *Saccharomyces cerevisiae* during apoptosis induced by H_2O_2

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We identified the proteins involved during apoptosis induced by H_2O_2 in *Saccharomyces cerevisiae*, and analyzed the global protein pattern by 2-DE. We analyzed classical parameters of apoptosis such as chromatin condensation, DNA fragmentation, and morphology changes of cells. Exposure of yeast cells to nonphysiological doses of peroxides decreases the expression (or increases degradation) of enzymes involved in protection against oxidative stress. This leads the yeast cells to a reduction of their antioxidant defense and makes the cells more prone to apoptosis. In our data the down expression of peroxiredoxin II and GST I, could induce a perturbation of mitochondrial function with an alteration of permeability of the membrane leading to the mitochondria-mediated apoptosis. Moreover, we identified a new spot of a classical glycolytic enzyme: the glyceraldehyde 3-phosphate dehydrogenase during apoptosis. It is known that GAPDH is an extremely abundant glycolytic enzyme with multiple functions and that its overexpression is evident during apoptosis induced by a variety of stimuli. Our results confirm that it is a major intracellular messenger mediating apoptotic death and that this new spot of GAPDH could be an intracellular sensor of oxidative stress during apoptosis induced by H_2O_2 in *S. cerevisiae*.

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1 Introduction

Apoptosis is a highly regulated form of cell death necessary during normal embryological development and in normal tissue homeostasis. This process is also important in the removal of mutated or infected cells and plays a role in pathogenesis of a variety of human diseases [1]. For many years apoptosis was assumed to be an exclusive metazoan process for its altruistic function, but several studies indicate that a form of programmed cell death, that is similar to mammalian apoptosis occurs in the unicellular organisms as bacteria and yeast *Saccharomyces cerevisiae* and recently there has been substantial evidence for apoptosis in fission yeast [2, 3]. Life and death of unicellular organisms belonging to communities are in some cases comparable to those of multicellular organisms. The budding yeast *S. cerevisiae* and the fission yeast *S. pombe* have been employed extensively for the study of a variety of complex pathways including cell division, secretion, and signal transduction. The finding of apoptosis in yeast is an important discovery *per se* and it discloses the possibility to use this simple organism as the model for apoptosis death occurring in higher eukaryotes.

Even if the *S. cerevisiae* genome does not contain any homologue of mammalian cell death executor genes, the expression of these mammalian genes, (ced-4, bax) induces cell death in yeast showing the phenotypic markers of apop-

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Abbreviations: c.f.u., colony-forming unit; **Prx,** peroxiredoxin

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tosis such as chromatin condensation and fragmentation, DNA breakage and exposition of phosphatidylserine. This is in accordance with the observation of chromatin condensation and nuclear fragmentation in *S. pombe* cells dying due to the expression of Bak [4] or CED-4 [5].

All these findings suggest that yeast contains a cell death mechanism related to a mammalian counterpart that can be activated by mammalian regulators. In *S. cerevisiae*, apoptosis can be detected after stimulation with acetic acid [6], H_2O_2 [7], UV [8] treatment and also during some physiological processes as aging and response to the α-factor [9, 10]. Madeo *et al.* [7] illustrated a central role of ROS in apoptosis in yeast, showing that the normal process of respiration in mitochondria is a major source of ROS and production of ROS is enhanced when mitochondrial function is disturbed during apoptosis.

More recently, the identification of an *S. cerevisiae* caspase-like protein Yca1p [11], Cdc48 [12], and Nma111p the yeast homologue of Omi/HtrA2 [13], that are involved in the apoptotic cascade, strengthens the claim that apoptosis occurs in yeast [14–18]. Other homologues of genes regulator of mammalian apoptosis have been identified in yeast, such as the yeast mitochondrial fission factor Fis1p [19] and the apoptosis-inducing factor Aif1p [20].

In spite of an increasing amount of data about *stimuli* that can induce apoptosis in yeast, little is known about the signaling pathways that are involved in the realization of the programmed cell death. Since the elements of the pathways are conserved in yeast as well as in mammals, yeast should be useful as a model to study these different pathways. The aim of the present study is to investigate the *S. cerevisiae* proteome during apoptosis induced by H_2O_2, in order to identify which pathways are more affected during this process. Since H_2O_2 was demonstrated to induce apoptosis in mammals as well as in yeast, we choose this kind of *stimulus* in order to obtain information about the proteins involved, useful to understand the apoptotic mechanism in higher eukaryotes. In this work we used a proteomic approach based on 2-DE and computer analysis to assess changes in patterns of yeast cells, combined with MS to identify interesting proteins.

2 Materials and methods

2.1 Yeast strain and growth conditions

The strain used in this study is W303-A (MATa leu2-3, 112ura3-1 trp1-92 his3-11, 15 ade2-1 can1-100, GAL SUC mal). Yeast cells were grown at 30°C in YEPD medium (1% yeast extract and 2% peptone and 2% w/v glucose used as carbon source). Solid media contained 2% w/v agar. To induce apoptosis, H_2O_2 was added to a final concentration of 3 mM to W303 cells exponentially growing (0.6–0.8 OD/mL) in YEPD and cells were collected after 3 h.

2.2 Cell viability assay

Cell viability was measured by plating serial dilution of treated and untreated cells on YEPD plates. The percentage of colony-forming units (c.f.u.) of treated cells was obtained by relating the c.f.u. counts of treated cells to those untreated which were considered to be 100%.

A trypan blue (Sigma, 0.4% solution, cat. T-8154) exclusion assay was adopted to assess cell viability during H_2O_2 treatment. Cells were washed with distilled water and stained with 0.01% trypan blue in water for 1 h. At least 200 cells from each sample were examined under the microscope to determine the proportion of stained cells.

2.3 Apoptotic markers

After 3 h of incubation with H_2O_2, apoptotic phenotype was controlled with TUNEL assay using the Roche Diagnostic *In Situ* Cell Death Detection Kit Peroxidase. Briefly, yeast cells were fixed for 1 h with 3.7% formaldehyde. The cell wall was digested with Lyticase (Sigma) and cells were applied to a polylysine-coated microscope slides. The slides were rinsed with PBS, incubated in blocking solution (3% H_2O_2 in methanol) and then in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) on ice for 2 min. The slides were rinsed twice with PBS and incubated with 20 μL of TUNEL reaction mixture (18 μL TUNEL label containing fluorescein-dUTP, 2 μL TUNEL enzyme) for 1 h at 37°C in a humid chamber in the dark. Slides were washed three times in PBS, and incubated with 50 μL of converted-POD and DAB substrate (Roche) precipitation was visualized under light microscope.

2.4 Preparation of yeast cell extracts by 2-DE

For 2-DE, apoptotic and control cells were harvested by centrifugation at room temperature. The pellet was washed twice in water and resuspended in 8 M urea, 4% CHAPS, and 10 mM DTT. Cells were broken with glass beads in a Fastprep instrument (Savant) and protein extracts were clarified by centrifugation at $8000 \times g$ for 10 min. For each experimental condition at least three samples were run in order to assess biological and analytical variation. IEF (first dimension) was carried out on nonlinear wide-range IPGs (pH 3–10; 18 cm long IPG strips; GE Healthcare, Uppsala, Sweden) and achieved using the Ettan™ IPGphor™ system (GE Healthcare). Analytical-run IPG-strips were rehydrated with 60 μg of total proteins in 350 μL of lysis buffer and 0.2% v/v carrier ampholyte for 1 h at 0 V and for 8 h at 30 V, at 16°C. The strips were then focused according to the following electrical conditions at 16°C: 200 V for 1 h, from 300 to 3500 V in 30 min, 3500 V for 3 h, from 3500 to 8000 V in 30 min, 8000 V until a total of 80 000 V·h was reached. MS-preparative IPG-strips were instead rehydrated with 350 μL of lysis buffer and 2% v/v carrier ampholyte, for 12 h at room temperature. Sample load, 800 μg *per* strip, was successively

performed by cup loading in the IPGphor Cup Loading Strip Holders (GE Healthcare), and, to obtain a successful focusing on basic pH values, it was applied at the anodic end of the strip. IEF was then achieved according to the following voltage steps, at 16°C: 30 V for 30 min, 200 V for 2 h, 500 V for 2 h, from 500 to 3500 V in 30 min, 3500 V for 5 h, from 3500 to 5000 V in 30 min, 5000 V for 4 h, from 5000 to 8000 V in 30 min, 8000 V until a total of 95 000 V·h was reached. After focusing, analytical and preparative IPG strips were equilibrated for 12 min in 6 M urea, 30% v/v glycerol, 2% w/v SDS, 0.05 M Tris-HCl, pH 6.8, 2%w/v DTE, and subsequently for 5 min in the same urea/SDS/Tris buffer solution but substituting the 2% w/v DTE with 2.5% w/v iodoacetamide. The second dimension was carried out on 9–16% polyacrylamide linear gradient gels (18 × 20 cm × 1.5 mm) at 40 mA/gel constant current and 10°C until the dye front reached the bottom of the gel, according to Laemmli [21] and Hochstrasser *et al.* [22]. Analytical gels were stained with ammoniacal silver nitrate as previously described [23]; while MS-preparative gels were stained according to a silver staining protocol compatible with MS [24]. Stained gels were digitalized using a Molecular Dynamics 300S laser densitometer (4000 × 5000 pixels, 12 bits/pixel; Sunnyvale, CA, USA).

2.5 Image analysis and statistics

For each strain condition namely control and apoptotic yeast cells, 2-D gels were run in triplicate and only spots present in all the replicates were taken into consideration for subsequent analysis. Computer-aided 2-D image analysis was carried out using the MELANIE 4.0 software (GeneBio, Geneva, Switzerland). Relative spot volumes (%V) (V = integration of OD over the spot area; %V = V single spot/V total spots) were used for quantitative analysis in order to decrease experimental errors. The normalized intensity of spots on three replicate 2-D gels was averaged and SD was calculated for each condition. A two-tiled nonpaired Student's t-test was performed using ORIGIN 6.0 (Microcal Software) to determine if the relative change was statistically significant. Reported pI and M_r (Da) values were experimentally determined by comigration with human serum as the internal standard [25]. CV was calculated as the SD expressed as a percentage of the mean.

2.6 Protein identification by MS

Protein identification was carried out by PMF on an Ettan MALDI-TOF Pro mass spectrometer (Amersham Biosciences), as previously described in Refs. [26, 27]. Electrophoretic spots, visualized by MS-compatible silver staining protocol, were manually excised, destained [28], and ACN dehydratated. Successively, they were rehydratated in trypsin solution, and in-gel protein digestion was performed by an overnight incubation at 37°C. From each excised spot, 0.75 μL of recovered digested peptides were prepared for MALDI-TOF MS by spotting them onto the MALDI target, allowed to dry, and then mixed with 0.75 μL of matrix solution (saturated solution of CHCA in 50% v/v ACN and 0.5% v/v TFA). After matrix application to the dried sample and its own drying, tryptic peptide masses were acquired. Mass fingerprinting search was carried out in NCBInr and Swiss-Prot databases using ProFound (http://129.85.19.192/profound_bin/WebProFound.exe), PeptIdent (http://www.expasy.org), and MASCOT (Matrix Science, London, UK, http://www.matrixscience.com) on-line-available softwares. Protein identification was achieved on the basis of corresponding experimental and theoretical peptide-fingerprinting patterns, using a peptide mass tolerance of 50 ppm, carbamylation modification of cysteine residues and allowing a single missed tryptic cleavage.

Tryptic digests that did not produce MALDI-TOF unambiguous identifications were subsequently acidified with 2 μL of a 1% TFA solution, and then subjected to ESI-IT MS/MS peptide-sequencing on a nanospray/LCQ DECA IT mass spectrometer (Thermo Finnigan, San Jose, CA, USA). Using the ZIP-TIP™ pipette tips for sample preparation (Millipore, Billerica, MA, USA), previously equilibrated in 50% ACN solution and abundantly washed in 0.1% TFA, acidified samples were enriched. Tryptic peptide elution from the ZIP-TIP™ matrix was achieved with a 70% methanol and 0.5% formic acid solution, and 3 μL of such concentrated sample solutions were then loaded in the nanospray needle. MS/MS database searching was performed by TurboSEQUEST (Thermo) and MASCOT MS/MS ion search software (http://www.matrixscience.com).

2.7 Western blot

After induction of apoptosis, 100 μg of protein extracts from control and apoptotic yeast cells were separated by 2-DE as previously described and transferred on PVDF membrane (Millipore). The blots were incubated with rabbit polyclonal GAPDH-HRP-conjugated antibodies (Abcam) in blocking buffer (PBS, 2% nonfat dry milk, 0.1% v/v Tween-20). The chemiluminescence immunodetection system (GE Healthcare) was used for signal development.

3 Results

3.1 Effect of H_2O_2 on apoptosis in *S. cerevisiae* W303

Previous reports from other authors [7] indicate that incubation of yeast cells with H_2O_2 3 mM for 200 min causes significant cell death by the induction of apoptosis. Before starting the differential proteome analysis which is the main purpose of this work, we wanted to obtain information about the time and H_2O_2 concentration-dependence study to induce apoptotic death in yeast cell strain W303. We used 3 mM H_2O_2 to induce apoptosis in exponentially growing yeast cells in complete medium compared with the control.

As shown in the Fig. 1A the percentage of survival, estimated by c.f.u. counts, decreases with time. At this concentration and after 3 h a loss of viability in about 90% of yeast cells evaluated by colony formation is evident; this is probably due to the high DNA damages accumulated that make cell replication impossible. Viability was also evaluated by trypan blue exclusion, a rapid and simple assay developed for the detection of dead cells. The method is based on the assumption that dead cells have nonfunctional membranes, through which the stain penetrates quickly, whereas living cells exclude the dye. Thus, dead cells stain, living cells remain colorless. Our results show that after 3 h of treatment with 3 mM H_2O_2, cells are not permeable to dye, this means that probably the cells retain some metabolic activities that permit them to exclude the dye (data not shown). All these data suggest that an apoptotic process could be the predominant cell death mechanism in treated cells. The apoptotic phenotype was then confirmed by TUNEL assay. After 3 h of treatment with 3 mM H_2O_2, about 80% of the cells show DNA cleavage as revealed in Fig. 1C. The arrows show some cells with TUNEL positive phenotype indicating the nuclear fragmentation with an occurrence of DNA strand breaks, in the treated cells (Fig. 1C) in comparison to the control (Fig. 1B).

3.2 Proteomic analysis

To investigate the *S. cerevisiae* protein expression during apoptosis, a comparative proteome analysis was performed using W303 cells treated with 3 mM H_2O_2, in comparison to nontreated cells. After 3 h, proteins were extracted, resolved by 2-D SDS-PAGE and the resulting silver stained electropherograms were analyzed using the MELANIE 4.0 software. In Fig. 2, typical 2-DE images of soluble yeast proteins from

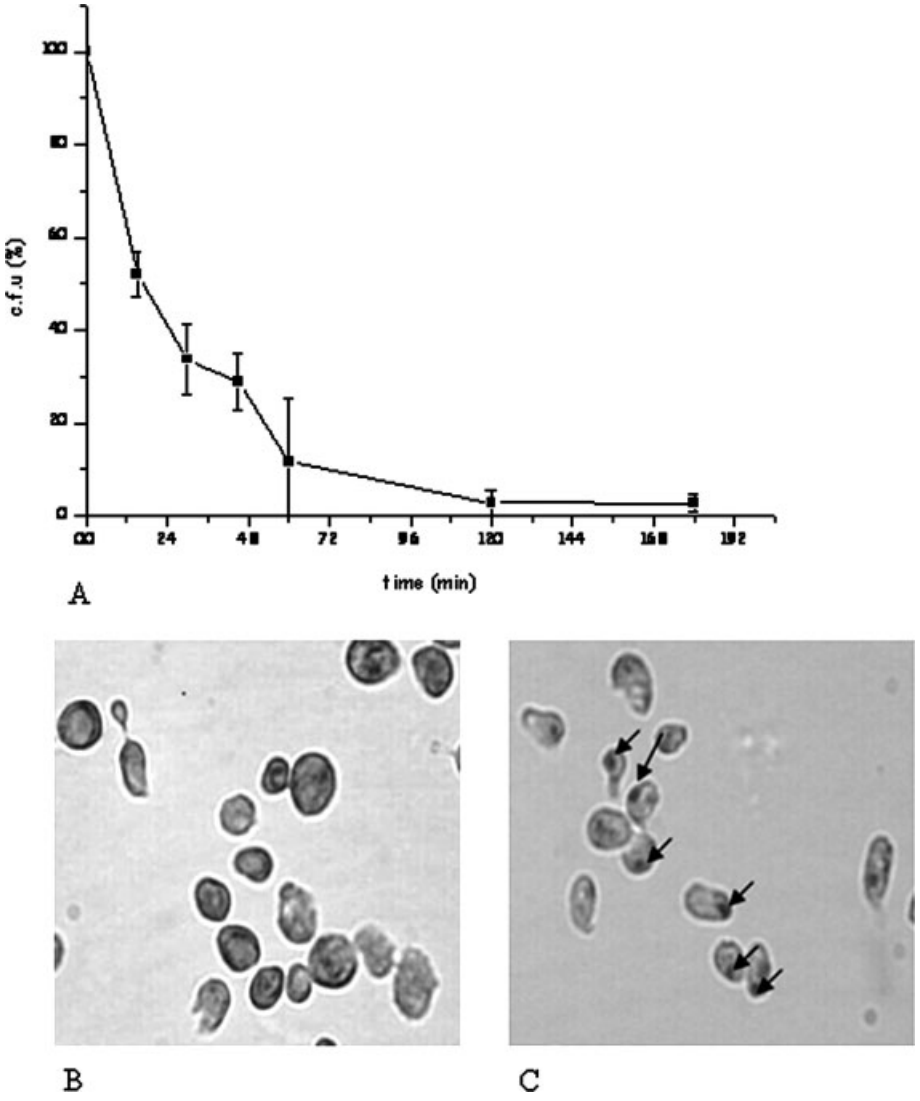
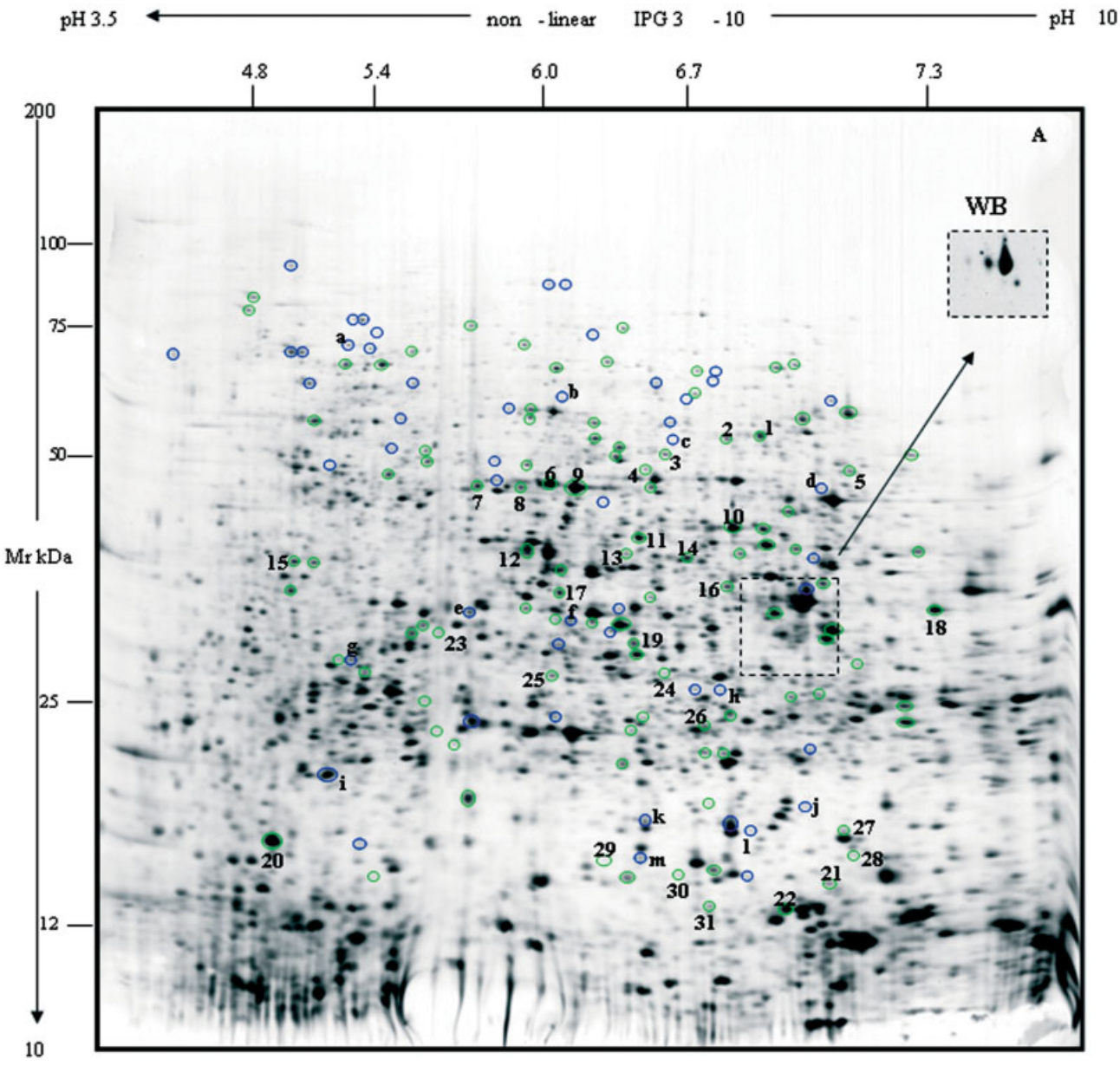


Figure 1. Apoptotic phenotype of H_2O_2 treated yeast cells. (A) Relative survival (% of c.f.u. on YEDP agar plates; 100% corresponds to the number of c.f.u. at time 0) of *S. cerevisiae* incubated with 3 mM H_2O_2 for 180 min. Vertical bars represent SD. TUNEL reaction to detect apoptotic phenotype. (B) Control strain; (C) *S. cerevisiae* after 180 min of treatment with 3 mM H_2O_2. Arrows indicate nuclear fragmentation.



control (panel A) and apoptotic cells (panel B) are shown. The pattern and relative abundance of proteins displayed correspond well with yeast 2-D protein profile produced previously in our laboratory [29]. An average of about 2000 spots were detected in each 2-D gel stained with silver. The quantitative and qualitative analyses were performed in triplicate and an expression change was considered significant if the intensity of corresponding spot differed reproducibly more than two-fold in relative volume (%*V*) between the two types of yeast cells and was statistically significant. This general pattern was conserved between replicate gels indicating the high degree of reproducibility of sample preparation and 2-D procedures. The computer analysis pointed out 102 quantitative variations between control and apoptotic cells (Fig. 2, green circles), while 51 (Fig. 2, blue circles) and 46 (Fig. 2, red circles) spots were exclusively detected in control or apoptotic cells, respectively. Identification of interesting proteins was carried out by MALDI-TOF MS and ESI-IT MS. Overall the results reported here indicate that induction of apoptotic death using H_2O_2, causes a significant alteration of protein expression profile in comparison to the untreated yeast cells.

3.3 Identification of differentially expressed proteins

Among the differentially expressed proteins a total of 31 spots were successfully identified by MS analysis selected on the basis of their relative abundance, high variation, and

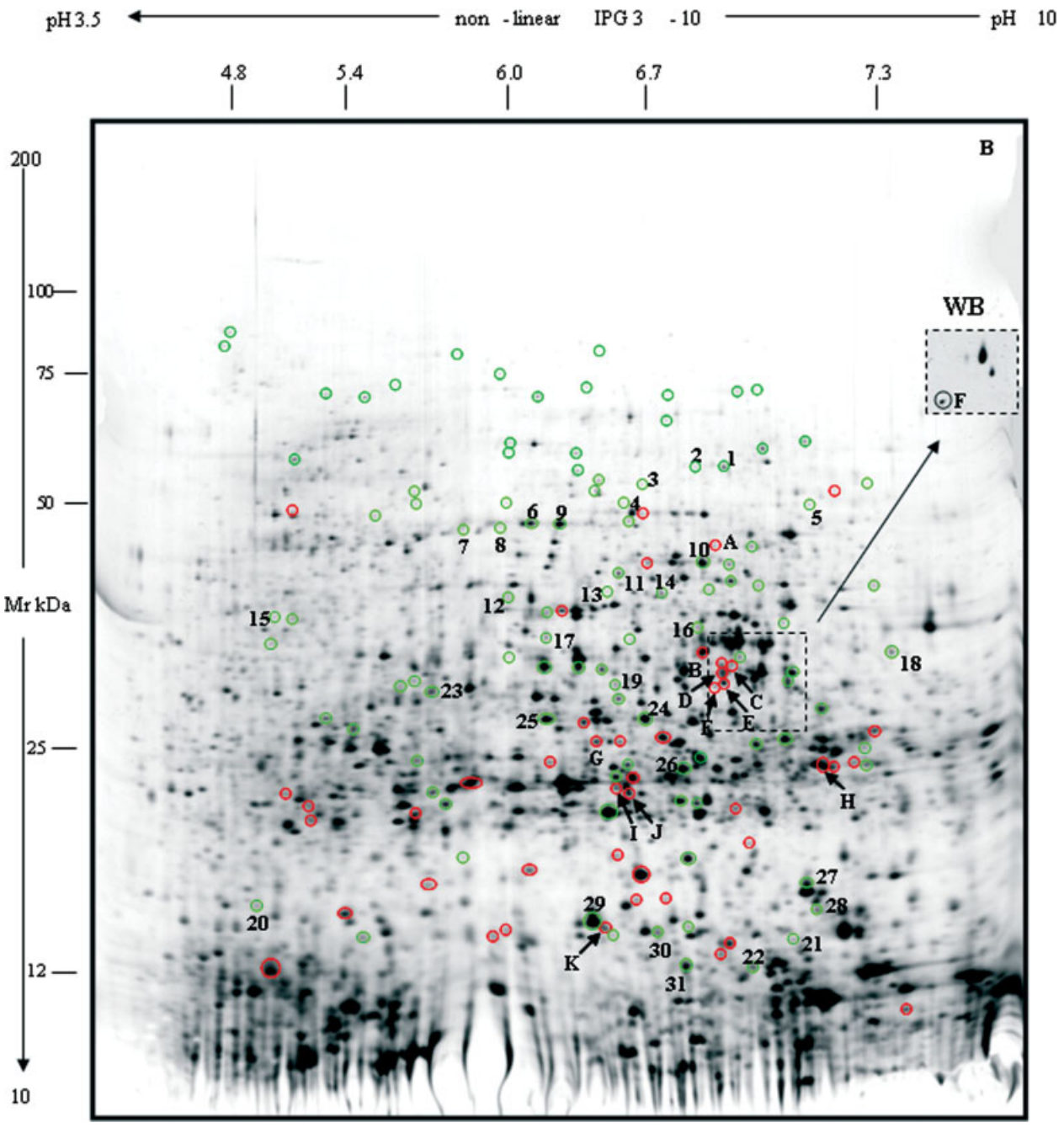


Figure 2. 2-DE silver-stained gels of total protein extract from control and H_2O_2 apoptotic cells. Quantitative variations between control (panel A) and apoptotic yeast cells (panel B) are displayed by green circles. Numbers identify proteins that are present in both gels but display a different expression level. Blue circles and red circles represent spots present exclusively in control and apoptotic cells, respectively. Small (control cells) and capital (apoptotic cells) letters indicate the identified proteins. Areas highlighted by the box illustrate the Western blot analysis identifying the new spot (F) of GAPDH present in the apoptotic yeast cells and absent in control cells.

resolution on preparative gels. The locations of these spots were marked with numbers in representative gels shown in Fig. 2 (panels A and B). For protein spots with low MASCOT score (spots 19, 22, and 28), ESI-IT MS/MS peptide-sequencing was performed to confirm protein identification. The identified proteins belonging to this category are described and their relative amounts are reported in Table 1. Twenty-one protein spot volumes change significantly at the 99% confidence level ($p<0.01$), one protein spot volume (spot number 28) changes significantly at the 98% con-

fidence level ($p<0.02$), six protein spot volumes (spots 4, 7, 12, 17, 24, and 26) change significantly at the 95% confidence level ($p<0.05$), while three protein spot volumes (spots 8, 15, and 23) show a relative change in expression significant at the 90% confidence level ($p<0.1$). The average CV of the differentially expressed spots is 22 and 24% for gels from control and apoptotic cells respectively indicating a typical variability according to Molloy *et al.* [30]. According to their biological activity, the proteins are sorted in different functional groups and their relative expression changes are reported in Fig. 3.

As shown in Table 1 and in corresponding Fig. 3, the comparative analysis revealed that apoptosis induces a down-regulation of the expression levels of a large number of the yeast proteins. The expression levels decrease in three protein spots as the apoptotic phenotype appears. The expression level of enolase (spots 6 and 9) and GAPDH (spot 18) falls as apoptosis proceeds. The down-regulation of these proteins is thought to reflect growth arrest which occurs to save energy to induce apoptotic phenotype. The presence of several protein fragments indicates that protein pattern variation associated to apoptosis induced by H_2O_2, involves an activation of proteolysis with the degradation of several proteins. Spots 23, 25, and 29 corresponding to fragments of enolase appear to increase significantly. Other spots showing increased abundance included spots 26–28 corresponding to fragment of GAPDH isoenzymes 2 and 3. These increases could be due to an enhanced rate of degradation of specific proteins during apoptosis, which can reflect the loss of protein function.

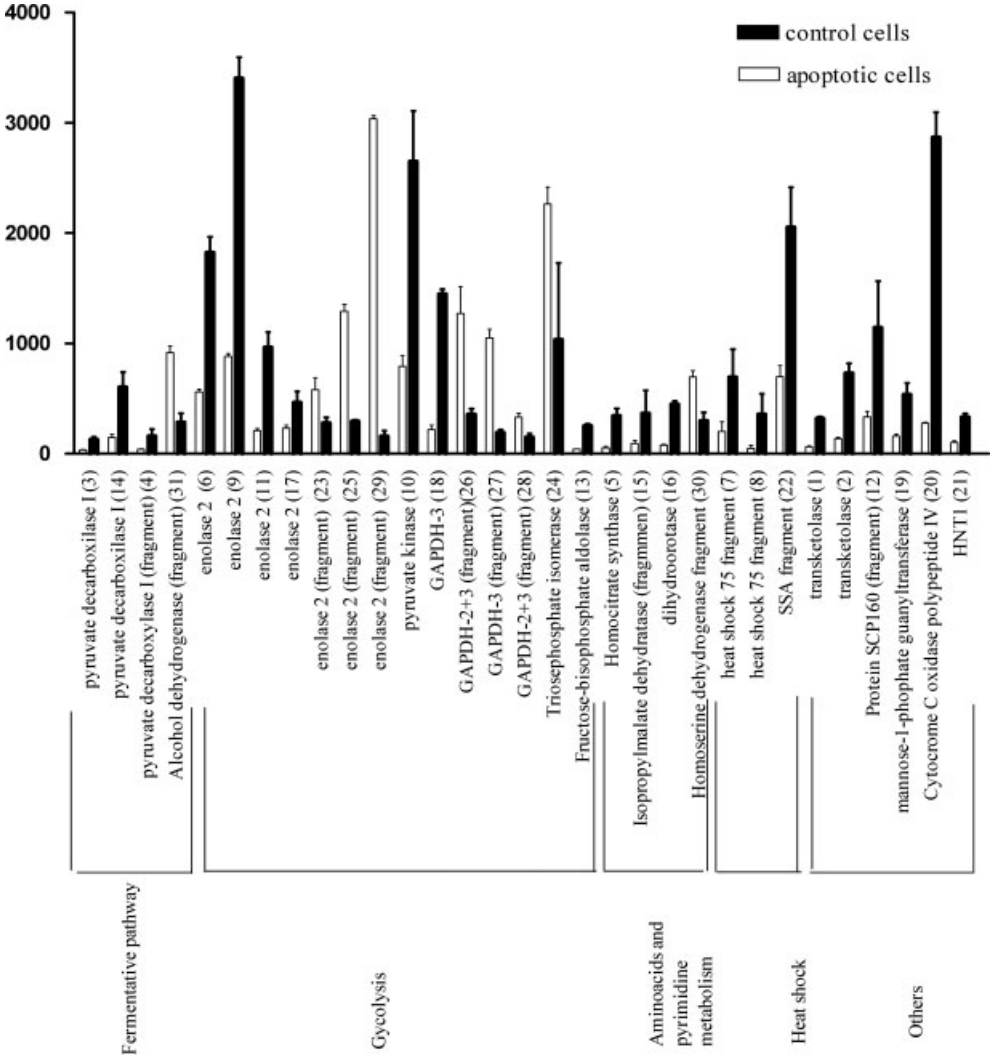


Figure 3. Relative protein expression changes of apoptotic *vs.* control cells. Relative expression changes of proteins that are affected by 3 mM H_2O_2. White bars (apoptotic cells) and black bars (control cells) represent the mean ± SD of computer volume percentage from three different control and apoptotic gels. $\%V\ (\times 10^{-4})$ is calculated as: V single spot/V total spots (V = integration of OD over the spot area). Proteins are grouped into five different categories: fermentative pathway, glycolysis, amino acids and pyrimidine metabolism, heat shock proteins, and others.

Table 1. Relative changes in protein expression in control *vs.* apoptotic cells

Spot no.	Protein name	AC[a]	Theoretical[b] p*I*/M_r (kDa)	Experimental[c] p*I*/M_r (kDa)	[d]	No. of matched peptides[e]	Sequence coverage[f] (%)	%*V* ($\times 10^{-4}$) mean ($\pm$SD)[g] Control	Apoptosis	*p* value[h] ($\times 10^{-5}$)	Fold[i] change c/a
1	Transketolase 1	P23254	6.5/73.67	6.7/54.02	71	8	15	321 ± 13	62 ± 14	3.28	5.2
2	Transketolase 1	P23254	6.5/73.67	6.6/53.34	150	12	21	731 ± 86	136 ± 23	32.6	5.4
3	Pyruvate decarboxylase 1	P06169	5.8/61.36	6.3/50.38	191	13	38	128 ± 25	33 ± 6	276	3.9
4	Pyruvate decarboxylase 1 (fragment)	P06169	5.8/61.36	6.3/47.74	154	13	31	160 ± 65	39 ± 13	3396	4.1
5	Homocitrate synthase cytosolic isozyme	P48570	6.84/47.10	7.0/47.42	160	12	35	344 ± 64	55 ± 20	178	6.3
6	Enolase 2	P00925	5.67/46.78	5.9/45.99	114	9	24	1824 ± 141	560 ± 40	11.9	3.3
7	Heat shock protein 75 (fragment)	P11484	5.32/66.47	5.6/45.71	107	11	24	693 ± 255	204 ± 149	4534	3.4
8	Heat shock protein 75 (fragment)	P11484	5.32/66.47	5.7/45.61	183	15	36	359 ± 184	99 ± 74	8633	3.6
9	Enolase 2	P00925	5.67/46.78	6.0/45.52	162	13	46	3405 ± 190	882 ± 46	2.38	3.9
10	Pyruvate kinase 1 (fragment)	P00549	7.56/54.54	6.6/41.35	88	9	26	2651 ± 456	793 ± 168	270	3.3
11	Enolase 2	P00925	5.67/46.78	6.2/40.37	147	10	29	964 ± 139	210 ± 33	79.6	4.6
12	Protein SCP160 (fragment)	P06105	5.64/134.81	5.8/39.19	62	9	9	1145 ± 420	334 ± 87	3050	3.4
13	Fructose-bisphosphate aldolase	P14540	5.51/39.49	6.2/38.82	102	9	38	253 ± 16	41 ± 5	2.89	6.2
14	Pyruvate decarboxylase 1 (fragment)	P06169	5.8/61.36	6.4/38.33	133	12	33	604 ± 135	149 ± 47	530	4
15	3-Isopropylmalate dehydratase (fragment)	P07264	5.61/85.79	4.8/37.72	61	6	10	366 ± 206	92 ± 51	8953	3.9
16	Dihydroorotase	P20051	6.07/40.313	6.6/34.75	162	7	24	450 ± 27	78 ± 14	2.34	5.8
17	Enolase 2	P00925	5.67/46.78	5.9/34.11	138	12	36	464 ± 101	232 ± 44	2248	2
18	Glyceraldehyde 3-phosphate-dehydrogenase 3	P00359	6.49/35.61	7.3/32.62	96	8	33	1447 ± 44	221 ± 64	1.06	6.5
19	Mannose-1-phosphate guanyltransferase	P41940	5.95/39.56	6.2/29.77		ETFPILVEEK[j]		537 ± 104	157 ± 36	404	3.4
20	Cytocrome *c* oxidase polypeptide IV	P04037	5.10/14.17	4.8/15.52	91	6	55	2869 ± 224	282 ± 26	3.8	10
21	HNT1	Q04344	6.44/17.68	6.9/13.53	82	6	51	331 ± 34	101 ± 25	73.1	3.3
22	Heat shock protein SSA2 (fragment)	P10592	4.95/69.34	6.7/12.45		DAGTIAGLNVLR[j]		2053 ± 364	699 ± 172	436	2.9
23	Enolase 2 (fragment)	P00925	5.67/46.78	5.5/30.62	110	13	33	275 ± 49	582 ± 195	6066	0.5
24	Triosephosphate isomerase	P00942	5.75/26.66	6.3/27.24	128	10	51	1035 ± 694	2262 ± 270	4682	0.5
25	Enolase 2 (fragment)	P00925	5.67/46.78	5.9/26.97	132	11	33	294 ± 127	1290 ± 110	10	0.2
26	Glyceraldehyde 3-phosphate dehydrogenase 2-3 (fragment)	P00358-9	6.49/35.71 6.49/35.61	6.5/23.19	68	6	23	357 ± 51	1269 ± 425	2099	0.3
27	Glyceraldehyde 3-phosphate dehydrogenase 3 (fragment)	P00359	6.49/35.61	7.0/16.15	86	8	32	189 ± 28	1050 ± 135	39.5	0.2
28	Glyceraldehyde 3-phosphate dehydrogenase 2-3 (fragment)	P00358-9	6.49/35.61	7.0/14.66		VAINGFGR[j] VLPELQGK[j]		149 ± 32	331 ± 65	1229	0.5
29	Enolase 2 (fragment)	P00925	5.67/46.78	6.1/14.42	72	7	17	159 ± 52	3037 ± 50	0.02	0.05
30	Homoserine dehydrogenase (fragment)	P31116	6.86/38.50	6.4/13.77	94	7	22	299 ± 78	696 ± 99	555	0.4
31	Alcohol dehydrogenase I (fragment)	P00330	6.26/36.69	6.5/12.59	65	6	23	322 ± 31	951 ± 94	34.6	0.3

a) Swiss-Prot/TrEMBL accession number.
b) Predicted p*I* and M_r according to protein sequence.
c) p*I* and M_r values were experimentally determined using human serum as internal standard.
d) MASCOT score (Matrix Science, London, UK; http://www.matrixscience.com).
e) Number of peptide masses matching the top hit from Ms-Fit PMF.
f) Percentage of amino acid sequence coverage of matched peptides in the identified proteins.
g) Each value represents the mean ± SD of individually computed %*V* (*V* = integration of OD over the spot area; %*V* = *V* single spot/*V* total spots) in three different gels of control and apoptotic cells.
h) A Student's *t*-test was performed to determine if the relative change was statistically significant.
i) Fold change (control *vs.* apoptosis) was calculated dividing %*V* from control by the %*V* from apoptotic gels.
j) Sequence tag identified by ESI-IT MS/MS peptide-sequencing.

3.4 Proteins exclusively detected in control yeast cells

Thirteen out of these 51 proteins, whose expression resulted in inhibition during the apoptotic process, were successfully identified (Table 2). The locations of these 13 protein spots are marked with small letters in the representative gel shown in Fig. 2A. In Fig. 4, are shown two examples of spots corresponding to proteins which disappear during apoptosis: peroxiredoxin (Prx) II and eukaryotic translation factor 4B.

Some of these proteins (spots h and i, respectively), are related to the cellular antioxidant defense and comprise a protein involved in the pathways for detoxification, the GST I and an antioxidant enzyme reducing hydroperoxides, the Prx II. This contains a conserved Cys residue that undergoes a cycle of peroxide-dependent oxidation and thiol-dependent reduction during catalysis. GST I has been shown to reactivate oxidized Cys of Prx. GST are ubiquitous in nature and this suggests their functions in living systems in addition to detoxification [31]. Recently, it has been demonstrated the formation of a dimer between GST with a Cys of Prx has been demonstrated. It has been hypothesized that Prx might be essential in protection of cells against apoptosis induced by a variety of stress-inducing agents [32]. Moreover, it has been found that Prx are essential proteins required for appropriate mitochondrial function. Of the 13 identified proteins one is involved directly in glycolysis: the fructose-bisphosphate aldolase, spot g. Others, identified as fragments, are involved in the fermentative pathway and in amino acid metabolism.

3.5 Proteins exclusively detected in apoptotic yeast cells

The identity of 11 of the 46 proteins induced during apoptosis is reported in Table 3. The locations of these 11 identified proteins are marked with capital letters in the representative gel shown in Fig. 2B. Many of the new spots induced by apoptosis and present only in the treated cells, belong to new fragments of several proteins (spots B and C both identified as fragments of phosphoglycerate kinase). It is interesting to observe that a glycolytic enzyme, the GAPDH isoform 3 (spot F), is present in the apoptotic cells as a new spot in the 2-D gel (Fig. 2) this new spot is shown in the enlargement in Fig. 4. The Figure shows another typical example of protein spot in which expression level is detectable only in apoptotic cells, the enolase isoform 2 (fragment). The glyceraldehyde-3-phosphate dehydrogenase is a classical glycolytic enzyme and is a multifunctional protein. The biological significance of this new migration is still unclear but it could be due to potential modifications of the protein occurring during apoptosis induced by H_2O_2. Moreover, many groups reported that GAPDH translocates to the nucleus under oxidative stressors [33].

Table 2. Identification of proteins exclusively detected in control yeast cells

Spot	Protein name	AC[a)]	Theoretical[b)] p*I*/M_r (kDa)	Experimental[c)] p*I*/M_r (kDa)	d)	No. of matched peptides[e)]	Sequence coverage[f)] (%)
a	Eukaryotic translation initiation factor 4B (eIF-4B)	P34167	5.17/48.52	5.1/74.33	104	8	22
b	Aminopeptidase Y	P37302	5.37/53.9	5.9/61.76		FGFTAVVIYDNEPK[g)]	
c	Seryl-tRNA synthetase cytoplasmic	P07284	5.8/53.31	6.4/53.17		ELVSCSNCTDYQSR[g)]	
d	Transketolase 1 (fragment)	P23254	6.5/73.67	6.9/45.52	64	9	14
e	Pyruvate decarboxylase 1 (fragment)	P06169	5.8/61.36	5.6/32.40	107	10	26
f	Heat shock protein 60 (fragment)	P19882	4.9/57.82	6.0/31.64	103	9	18
g	Fructose bisphosphatealdolase	P14540	5.51/39.49	5.1/28.48	64	7	31
h	GST I	P40582	6.18/26.79	6.5/25.82	106	7	35
i	Prx type II	P38013	5.01/18.98	5.0/20.08	126	9	68
j	Glyceraldehyde 3-phosphate dehydrogenase 2	P00358	6.49/35.71	6.8/17.74		VLPELOGK[g)]	
k	Phosphoglycerate kinase (fragment)	P00560	7.09/44.61	6.3/16.76	70	6	17
l	Phosphoglycerate kinase (fragment)	P00560	7.09/44.61	6.6/16.16	75	6	22
m	60S ribosomal protein L27	P38706	15.53/10.36	6.2/14.52		1) NQWFFSK[g)] 2) VVNYNHLLTR[g)]	

a) SwissProt/TrEMBL accession number.
b) Predicted p*I* and M_r according to protein sequence.
c) p*I* and M_r values were experimentally determined using human serum as internal standard.
d) MASCOT score (Matrix Science, London, UK; http://www.matrixscience.com)
e) Number of peptide masses matching the top hit from Ms-Fit PMF.
f) Percentage of amino acid sequence coverage of matched peptides in the identified proteins.
g) Sequence tag identified by ESI-IT MS/MS peptide-sequencing.

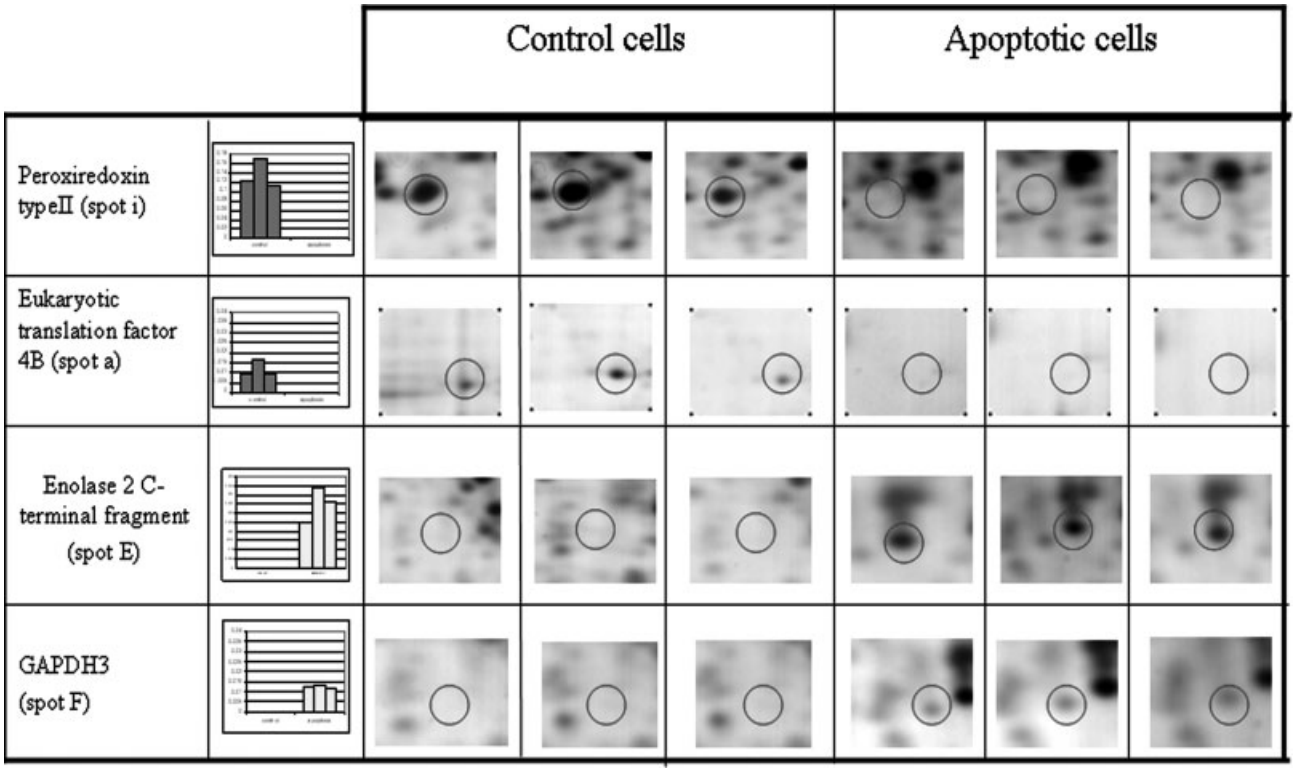


Figure 4. Magnified regions of triplicate 2-D gel images of proteins only detected in control or in apoptotic cells. Protein spots of interest are indicated with circles, with the corresponding protein names pointed out on the left. Gel segments shown in this figure were enlarged from Fig. 2. Histograms show the protein abundance, with protein intensity of the 2-D gels indicated in gray and white, for control and apoptotic cells, respectively.

Table 3. Identification of proteins exclusively detected in apoptotic yeast cells.

Spot	Protein name	AC[a)]	Theoretical[b)] p*I*/M_r (kDa)	Experimental[c)] p*I*/M_r (kDa)	d)	No. of matched peptides[e)]	Sequence coverage[f)] (%)
A	Transketolase 1 (fragment)	P23254	6.5/73.67	6.6/43.77		EALDFQPPSSGSGNYSGR[g)]	
B	Phosphoglycerate kinase (fragment)	P00560	7.09/44.61	6.6/33.13	117	8	25
C	Phosphoglycerate kinase (fragment)	P00560	7.09/44.61	6.7/31.99	108	7	22
D	Protein SCP160 (fragment)	P06105	5.64/134.80	6.6/31.25	88	8	7
E	Enolase 2 (fragment)	P00925	5.67/46.78	6.7/30.17	83	8	19
F	Glyceraldehyde 3-phosphate dehydrogenase 3	P00359	6.49/35.61	6.6/29.82	123	11	40
G	Enolase 2 (fragment)	P00925	5.67/46.78	6.1/26.43	85	7	20
H	Elongation factor 1 − α (fragment)	P02994	9.03/50.14	7.0/24.22	81	6	21
I	Triosephosphate isomerase	P00942	5.75/26.67	6.2/22.42	137	8	39
J	Heat shock protein SSA2 (fragment)	P10592	4.95/69.34	6.3/22.06	93	9	18
K	Enolase 2 (fragment)	P00925	5.67/46.78	7.0/14.13	75	6	15

a) Swiss-Prot/TrEMBL accession number.
b) Predicted p*I* and M_r according to protein sequence.
c) p*I* and M_r values were experimentally determined using human serum as internal standard.
d) MASCOT score (Matrix Science, London, UK; http://www.matrixscience.com).
e) Number of peptide masses matching the top hit from Ms-Fit PMF.
f) Percentage of amino acid sequence coverage of matched peptides in the identified proteins.
g) Sequence tag identified by ESI-IT MS/MS peptide sequencing.

3.6 Western-blot analysis of GAPDH protein expression in H_2O_2 apoptotic cells

In order to validate the results discussed above in which we identify by 2-D and MS a new spot corresponding to a GAPDH isoform (spot F in Fig. 2B), we analyzed the proteins extracted from apoptotic or nonapoptotic cells by Western blotting with anti-GAPDH antibodies. Our results reveal that the new GAPDH isoform present in apoptotic cells (Fig. 2 WB) is not visible in the Western blot from the control cells. The results shown in the 2-D Western blotting of GAPDH are consistent with the results from 2-DE and MS-based identification. The new spot F, corresponding to GAPDH isoform by MS identification, is evident in the immunoblots derived from extracts of apoptotic and nonapoptotic cells (Supporting Information Fig. 1).

4 Discussion

The yeast model could help to understand the complicated hierarchy between different apoptotic pathways and be able to elucidate the protein pathways involved in apoptosis. In this study, we investigated the changes in the proteome of yeast *S. cerevisiae* to identify proteins that are implicated in apoptosis induced by H_2O_2 and we verified that this treatment induces many changes in the expression level of proteins involved in metabolism, stress response, and oxidant protection.

A general down regulation of expression level of enzymes involved in glycolysis, fermentation, and other metabolic functions is observed. Since after H_2O_2 treatment the cells stop dividing, the global decrease in protein expression verified with proteome analysis, could be attributed to a general inhibition of protein synthesis and/or an increase in protein degradation. Protein synthesis could be impaired since some proteins involved in this process are detectable only in control cells and on the other hand, during apoptosis there is an increase in several fragments of metabolic enzymes that could be the result of a partial degradation. We identified two proteins in which the expression is dramatically reduced in the apoptotic cells: the GST I and Prx II. These are implicated in protection against oxidative stress, and are potentially involved as antiapoptotic proteins [34]. Hydrogen peroxide and other ROS, play a role key in a variety of biological processes but can damage nucleic acids, proteins, and lipids. Therefore, specific cell antioxidant systems are essential to control the intracellular level of ROS. These systems include reducing agents like glutathione and thioredoxin. Prx reduce hydrogen peroxide by electron transfer from thioredoxins, glutathione, or cyclophilins. Moreover, in the cells the reduction pathway involves glutathione and GST. In our results, exposure of yeast cells to nonphysiological doses of peroxides, significantly decreases the abundance of these enzymes. All these data suggest that H_2O_2 treatment decreases the expression (or increases degradation) of these enzymes leading the yeast cells to a reduction of their antioxidant defense and a down regulation of the expression of these proteins makes the yeast cells more prone to apoptosis. It has been recently shown that adaptation of cells to H_2O_2 is in part due to the redirection of carbohydrate flux from glycolysis to the pentose phosphate pathway to the regeneration of NADPH responsible for the maintenance of both Prx and glutathione in their reduced states [35]. It is well known that, in mammalian cells, Prx are involved in the regulation of several mitochondrial processes such as an alteration of mitochondrial structure. In our data the down expression of Prx II during H_2O_2 treatment, could induce a perturbation of mitochondrial function bringing about an alteration of permeability of the membrane leading to the mitochondria-mediated apoptosis. Numerous studies demonstrated that survival and cell death are linked to glucose metabolism [33] and that apoptosis is partly dependent on energy status. In our results it is interesting to note that two glycolytic enzymes increase their expression level in comparison to the control: triosephosphate isomease and GAPDH (isoform 3). Since these enzymes work in sequence during glycolysis, it is possible to suggest a maintenance of glycolysis for the production of energy within cells. In particular, we can observe a new spot of GAPDH isoform 3 in the proteome from apoptotic yeast cells. GAPDH is an extremely abundant glycolytic enzyme with multiple and seemingly unrelated functions. Recent studies demonstrated that GAPDH expression was increased during apoptosis induced by a variety of factors including nutrient starvation, prolonged culture, and cytotoxic agent treatment [36]. In conclusion our results suggest that this new spot of GAPDH could be an intracellular sensor of oxidative stress during apoptosis induced by H_2O_2 in *S. cerevisiae*.

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5 References

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