BioMetals **13:** 251–259, 2000.

Biochemical responses in a *Candida famata* strain adapted to high copper concentrations

Marisa Manzano[1,*], Luca Cocolin[1], Barbara Citterio[2], Lanfranco Conte[1], Marco de Bertoldi[1], Giuseppe Comi[1], Gianfranco Santovito[3], Mariano Beltramini[3] & Benedetto Salvato[3]
[1]*Dipartimento di Scienze degli Alimenti, Universitá di Udine, Via Marangoni 97, 33100 Udine, Italia*
[2]*Istituto di Igiene Tossicologica ed Ambientale, Universitá di Urbino, via S.Chiara 28, 61029 Urbino, Italia*
[3]*Dipartimento di Biologia, Universitá di Padova, via Ugo Bassi 58/B, 35131 Padova, Italia*
[*]*Author for correspondence: Department of Food Science, Via marangoni 97, 33100 Udine, Italy (Phone: 39 0432 590746/711; Fax: 39 0432 590719; E-mail: marisa.manzano@DSA.uniud.it)*

Received 7 July 2000; accepted 25 July 2000

Key words: aminoacids, capillary gas chromatography, copper, superoxide dismutase, yeasts

Abstract

A strain of *Candida famata* was adapted to high copper concentration (1.26 mM) and a number of biochemical parameters have been tested, in order to get information on the mechanisms of metal toxicity and detoxification as well as on the metabolic responses to the treatment. The cytoplasmic levels of superoxide dismutase, peroxidase and glutathione were found significantly increased with respect to control cells, in contrast to catalase which is not affected. The activities of enolase and of triosephosphate isomerase are found to decrease as a consequence of the exposure to copper. Statistically significant differences in the content of some aminoacids are found between copper-treated and control cells.

Introduction

Some metal ions, like copper ions, are considered essential for life. Other metals, for which a precise role in the metabolism so far has not been recognised, are considered toxic elements. However, also the essential metals, when present in excessive concentration, could have a cytotoxic effect (Stadtman 1990, Stadtman & Oliver 1991).

Once inside the cells, the metal ions are bound to cytoplasmic proteins so that the free metal concentration is kept low. The mechanisms for limiting metal toxicity are essentially three: the binding to peptides and proteins, the most important of which are metallothioneins; the compartimentation in membrane vesicles like lysosomes; the formation of insoluble precipitates like concretions of calcium and magnesium salts or granules of calcium and sulphur compounds (Viarengo & Nott 1993).

Among the complexes that copper may form with substrates and metabolites, particular importance is attributed to the glutathion (GSH) complexes. This tripeptide (Lys-Gly-Glu) is involved as protective agent against the toxic effects of copper by direct binding Cu(I) or by scavenging free-radicals produced from redox reactions catalyzed by redox active metal ions. In rat hepatoma cells, copper is complexed by GSH immediately after entering the cell (Steinebach & Wolterbeek 1994); copper may be transferred to metallothioneins where it is stored or could be eliminated from cells as GSH-complex (Freedman *et al.* 1989; Cousins 1985).

In this work we have studied the effects of copper uptake in *Candida famata* adapted to 1.26 mM copper in the culture medium and the mechanisms involved in the protection from the toxic effects of this metal. Since cells can modify the quantity or the quality of one or more molecules inside the cell to overcome some stressing factors, such as the addition of a substance like copper to the medium of growth and since the copper-dependent stimulation of metallothionein

synthesis (Butt & Ecker 1987; Etcheverry *et al.* 1986; Karin *et al.* 1984; Tohoyama *et al.* 1995) in yeast is well documented, our interest was to study the involvement of other cellular ligands such GSH, and the effects of copper administration on the enzymes related to the detoxification pathway of the activated oxygen species (superoxide dismutase, catalase, peroxidase) activity. Furthermore, the changes brought about by copper on the levels of various enzymes realted to the oxidative metabolism and on the cytoplasmic concentration of aminoacids was also studied. For these latter measurements a procedure based on gas-chromatographic analysis of aminoacids after their derivatisation was introduced in order to overcome the problems related to their low concentrations in the samples.

Materials and methods

Yeast strain and media

A *Candida famata* strain identified with the API ATB 32 IC kit as recommended by the manufacturer (Biomerieux SA, Marcy l' Etoile, France), was used. Cells were grown in Yeast Nitrogen Base (Difco, Detroit, Michigan, USA) liquid medium with 2% (w/v) glucose for the control condition (YNBG) and in YNBG with the addition of copper (as $CuSO_4 \cdot 5H_2O$, $CuCl_2$) and Na_2SO_4, both 1.26 mM final concentration.

Glucose, and salt solutions were sterilised by filtration with Minisart filter of 0.2 μm pore size (Sartorius, Gottingen, Germany) and added to the YNBG media previously autoclaved at 121 °C for 15 min. Cells for the measurement of growth rates were made active in a culture tube containing 10 ml of the media and were incubated at 30 °C for 24 h in an orbital shaker. They were inoculated (about 5×10^3–10^4 cells/ml) in flasks containing 100 ml of the media and kept in the same conditions. At regular intervals a count using a hemocytometer was made.

Cells, at the beginning of the stationary phase, were collected by centrifugation at 3300 *g* at 15 °C for 15 min using a refrigerated centrifuge (ALC mod. 4233 equipped with a RCF Meter) and used for biochemical analysis.

Preparation of the cell extract

Cells were resuspended in 5 ml of 20 mM sodium phosphate buffer pH 7.4 and disrupted using a vibrator apparatus (Biospec Products, Bartelesville, OK) with 0.45–0.50 mm (diameter) glass beads for 5 min. The cell lysis was verified by measuring the suspension turbidity (OD610) before and after the treatment (Rose & Veazey 1988). For a quantitative recovery of the lysate, the glass beads were washed three times with 5 ml of buffer. Cell omogenate and the buffer solutions used to rinse the disruption chamber were collected and pooled together. The lysate was then centrifuged at 35,000 *g* for 30 min (Beckman, Palo Alto, California, USA) and the supernatant was tested for biochemical determinations. For GSH determination, a part of cell extract was added to an identical volume of trichloroacetic acid (TCA) 0.6 M and re-centrifuged at the previously described conditions.

Biochemical determinations

Superoxide dismutase (SOD), catalase and peroxidase activities were assayed using a Lumicon (Hamilton, Bonaduz, Switzerland) luminometer. The method used for SOD was a modification of the method proposed by Puget & Michelson (1974), as described by Sarais *et al.* (1994). Catalase and peroxidase activities were determined using a modification of the method of Michelson *et al.* (1977), as described by Sarais *et al.* (1994), and according to Puget *et al.* (1977), respectively.

Total GSH concentration was estimated spectrophotometrically using a modification of the method of Anderson (1985). The sample was brought to 200 μl with milli Q water (Millipore, Milan, Italy) and incubated with 700 μl of NADPH (0.3 mM in 143 mM Na_2PO_4/6.3 mM Na_4-EDTA, pH 7.5) and 100 μl of DTNB (5,5′-dithiobis-(2-nitrobenzoic acid), 6 mM) in a cuvette for 15 min at 30 °C. After incubation, 44.3 μl of GSSG reductase (6 U/mg, Boehringer) was added in the sample-containing cuvette and the value of absorbance at 412 nm was estimated after 6 min. For each determination, a blank was made by substituting the sample with the same volume of TCA (trichloroacetic acid, 0.3 M). The GSH concentration was referred to a calibration curve obtained using aliquots of a 50 μM GSH (Sigma) standard solution.

The enzymatic assay for the enzymes of the glycolytic and pentose phosphate pathway was performed as described in Beutler (1984) with slight modifications for the hexokinase assay (Stocchi *et al.* 1994).

Protein concentrations were determinated according to the method of Lowry *et al.* (1951) with bovine serum albumin as standard. An Akontron Spectropho-

tometer (Model Uvikom 860) was used for optical measurements.

Aminoacid determinations

Preparation of the cell extract

After activation, yeast cells were inoculated in three flasks containing 20 ml of YNBG, incubated for 24 h at 30 °C under shaking. Broth cultures were centrifuged at 5000 × *g* for 15 min at 4 °C and the supernatant was discarded.

Cell lysis was made by the addition of 0.5 ml of a 1 M sorbitol – 0.1 M EDTA (pH 7.5) solution containing the lytic enzyme from *Rhizoctonia solani* (Sigma-Aldrich) (25 mg/ml) to the pellet.

The sample was homogenized, kept under shaking in a water bath at 45 °C for 2 h and centrifuged at 8,000 × *g* for 10 min. Then 0.5 ml of 50 mM Tris-HCl 20 mM EDTA pH 7.4 and 0.05 ml SDS 10% (pH 7.2) were added to the pellet and incubated in a water bath at 65 °C for 30 min. Finally, 0.2 ml of K-acetate 5 M were added and the sample was stored in ice for 30 min before centrifugation at 14,000 × *g* for 5 min at 4 °C. The clear solution obtained was subjected to purification as described by Adams (1974).

Derivatisation of aminoacids

The aminoacids (AA) were converted to their N-heptafluorobuthyl derivatives as described by Mac Kenzie & Teneschuk (1974) but with the modifications (time and temperature) as described by De Ming (1989).

GC analyses

Reagents

HFBA was obtained from Fluka, Buchs, Switzerland, Methylene chloride and isobutanol were of analytical grade (Carlo Erba RPE ACS, Milano, Italy) and were redistilled before use. The HCl/Isobutanol solution was prepared in the laboratory, by bubbling HCl in cold isobutyl alcohol, until a 3 M solution was obtained. The cationic exchange resin Dowex 50 × 8-200 (Aldrich) was used. Solutions of hydrocloric acid (1 M) and ammonium hydroxyde (2 M and 7 M) (Carlo Erba, Milan, Italy) were used.

Aminoacids clean up

Amino acids were extracted from the clear solution obtained as described by Adams (1974), with some modification, in order to optimize the clean up procedure for our samples. The internal standard (norleucine, 0.045 mg) was added to samples before transfer into the chromatographic column, prepared with 1 g of ion exchange resin, previously washed with distilled water until neutral washing water was obtained. A first elution to eliminate sugars was carried out with 12 ml of acid solution (water at pH 2.3), and the AA were eluted with 30 ml of ammonium hydroxyde 2 M.

Gaschromatographic analyses were performed using a Carlo Erba Mega 5160 instrument, equipped with an Electron Capture Detector (ECD 400 Carlo Erba Instruments, Milan, Italy) and a 3396A Hewlett Packard integrator (Avondale, P.A., USA). A capillary column CP SIL 8 CB (Chrompack; Belgium) 25 m × 0.25 mm i.d., 0.20 μm film thickness was used. The carrier gas was helium at 1 ml/min, nitrogen (auxiliary gas) was at 30 ml/min. The injector and detector temperature was 300 °C with splitting system 1:50. The initial isothermal was 2 min at 70 °C, then the temperature increment was: from 70 °C to 190 °C with 5 °C/min incremental rate, from 190 °C to 290 °C with 7.5 °C/min incremental rate and 20 min of final isothermal at 290 °C. A volume of 0.5 μl of the derivatised sample was injected.

A commercially available mixture of aminoacids from Sigma (St. Louis, Mo, USA) was used. An internal standard solution of norleucine (11.14 mg) was dissolved in 10 ml distilled water while the standard solution of aminoacids in 10 ml of NH_4OH 2N consisted of: alanine, glycine, valine, threonine, serine, leucine, norleucine (Internal Standard), proline, hydroxyproline, methionine, asparagine/aspartic acid (Asx), phenylalanine, glutamine/glutamic acid (Glx), lysine, tyrosine, arginine 10 mg each. Derivatisation was performed on single aminoacids and on their mixture.

For the determination of the response factor, ten aliquots of the standard mixture of the aminoacids were analysed in the same conditions used for the cells and injected. The response factor (K) was then calculated for each aminoacid using the following formula:

$$K = (W_{is}\ A_x)(W_x\ A_{is})^{-1}$$

where K is the response factor; W and A stand for the weight and peak area respectively. The subscripts x and is refer to the x-th aminoacid and the internal standard respectively.

Table 1. Level of SOD, catalase, peroxidase and glycolitic and pentose phosphate pathway enzymes in the control conditions (TQ) and in the copper treated cells (Cu80). The values reported in the table are the means ± Standard Deviation(SD) of three independent determinations. Values are expressed as U/mg proteins.

Enzymes	TQ		Cu80	
	mean	S.D.	mean	S.D.
SOD	18.9	± 5.6	172.4	± 24.6
Catalase	34.6	± 5.5	22.87	± 2.78
Peroxidase	222	± 30	419	± 80
Hesokinase	0.29	± 0.10	0.18	± 0.05
Phosphoglucose isomerase	0.39	± 0.09	0.27	± 0.01
Phosphofructokinase	0.044	± 0.020	0.021	± 0.010
Aldolase	0.013	± 0.005	0.009	± 0.001
Triosephosphate isomerase	1.32	± 0.02	0.66	± 0.20
Glyceraldehyde 3-phosphate dehydrogenase	0.44	± 0.09	0.27	± 0.06
Phosphoglycerate kinase	2.58	± 0.80	1.55	± 0.20
Phosphoglyceromutase	0.45	± 0.30	0.56	± 0.30
Enolase	0.81	± 0.01	0.42	± 0.10
Pyruvate kinase	0.69	± 0.20	0.55	± 0.06
Glucose 6-phosphate dehydrogenase	0.13	± 0.03	0.08	± 0.02
Lacticdehydrogenase	0.004	± 0.003	0.006	± 0.002
6Phosphogluconate dehydrogenase	0.074	± 0.020	0.042	± 0.004
Phosphoglucomutase	0.072	± 0.020	0.064	± 0.030

Method repeatability assessment

10 aliquots of AA standard solution (50 μl each) were withdraw to evaluate the repeatability of the method. Standards were derivatized individually and in association to control their retention times.

Statistical analyses

The statistical analyses of the data related to the aminoacids was made utilizing the Systat program (Wilkinson 1990).

Results

The growth curves of *Candida famata* determined in the absence and in the presence of 1.26 mM Cu (as $CuSO_4$) are reported in Figure 1. In the absence of Cu (control condition, TQ) the cells reach the stationary phase in 39 h. The presence of Cu in the culture medium increased the lag-time and about 56 h are required to reach the stationary phase.

The results of the determination of the activity of the enzymes involved in the glycolitic and pentose phosphate pathway are summarised in Table 1, together with the levels of SOD, catalase and peroxidase. As compared with the control cells, significant decreases of activity (normalised per mg of total proteins) are observed in the case of enolase ($P < 0.01$) and of triosephosphate isomerase ($P < 0.01$). The variations of the other enzymes tested are not statistically significant.

The concentration of SOD in cells adapted to 1.26 mM Cu (80 μg/ml, Cu80 cells) is about one order of magnitude higher than in control cells ($P < 0.001$) whereas the differences in catalase activity are scarcelly statistically significant ($P < 0.010$).

The total glutathione levels significantly increase in the case of Cu80 *Candida* cells as compared to TQ (Table 2) and the effect is clearly dependent on the copper salt used in the treatment, the effect being maximal with copper sulphate. It is worthnoting that the reinoculum of Cu80 cells, collected in the stationary phase, in a copper-deficient medium brings the total glutatahion levels back to the levels of TQ.

The total aminoacid pool was quantitatively and qualitatively assayed by a gas-chromatography technique after purification and derivatization. In Figure 2, a trace of AA standard solution (A) and the one

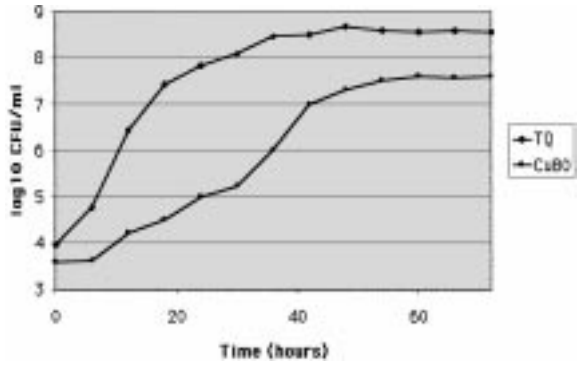

Figure 1. Growth curves of *Candida famata* in the absence and presence of $CuSO_4$ 1.26 mM (80 μg/ml).

Table 2. GSH level in yeast cells after the addition of different salts to the culture media (Yeast Nitrogen Base with Glucose, YNBG). Values are expressed as μg/mg proteins.

Strains	media	μg GSH/mg ptoteins
Control	YNBG	1.48 ± 0.31
Copper adapted	YNBG	1.57 ± 0.36
Copper adapted	YNBG+$CuCl_2$*	1.88 ± 0.16
Copper adapted	YNBG+Na_2SO_4*	2.58 ± 0.42
Copper adapted	YNBG+$CuSO_4$*	5.13 ± 1.07

*80 μg/ml final concentration.

of the samples (B and C) obtained by ECD detection, are reproduced. The gaschromatograms obtained were satisfactory and, although the presence of other materials is suspected by the presence of some spurious peaks, the AA quantification is possible. As far as the method reproducibility is concerned, this was assessed by replicating the analytical procedure ten times and evaluating the values obtained for the response constant. Data are reported in Table 3.

Considering the low concentration of free aminoacids in the cells, the high sensitivity of the method allowed the analyses of the cells grown in 20 ml of broth culture omitting any concentration step. The results of aminoacid determinations on cell extracts obtained from copper sulphate treated *Candida famata* cultures and controls are reported in Table 4. The levels of several aminoacids, such as glycine, valine, serine, leucine, hydroxyproline, methionine, aspartic acid and asparagine, phenylalanine, lysine and tyrosine are found to increase ($P < 0.001$–$P < 0.05$), whereas the content of alanine decreases upon copper treatment ($P < 0.01$). The difference between Cu80 and TQ cells are not significant in the case of threonine, proline, glutamate and glutamine.

Table 3. Response constant and percent coefficent of variation

Aminoacid	K (mean of ten replicates)	% RSD
Alanine	1,42	11,30
Glycine	1,92	13,42
Valine	0,80	9,46
Threonine	6,90	12,88
Serine	6,04	11,55
Leucine	0,93	3,75
Isoleucine	0,71	6,17
Proline	1,60	3,85
Hydroxyproline	6,00	11,55
Metionine	1,96	5,64
ASX*	1,67	8,59
Phenylalanine	1,38	5,07
GLX*	1,39	6,10
Lysine	3,22	13,37
Tyrosine	7,22	12,33
Arginine	2,37	9,02
Tryptophane	2,30	12,54
Cysteine	5,73	7,05

*ASX and GLX indicates, respectively the sum of Asparagine + Aspartic acid and of Glutamine + Glutamic acid, as after derivarisation, they gave the same structure.

Discussion

The increased level of SOD activity upon copper treatment is a clear metal-related response that depends on a co-stimulation of SOD and metallothionein genes by the same copper-activated cytoplasmic ACE1 activating protein factor (Thiele 1988). The activity of catalase is expected to be coupled to that of SOD in that the former enzyme removes the potentially toxic hydrogen peroxide produced by SOD (Bilinski & Litwinska 1987).

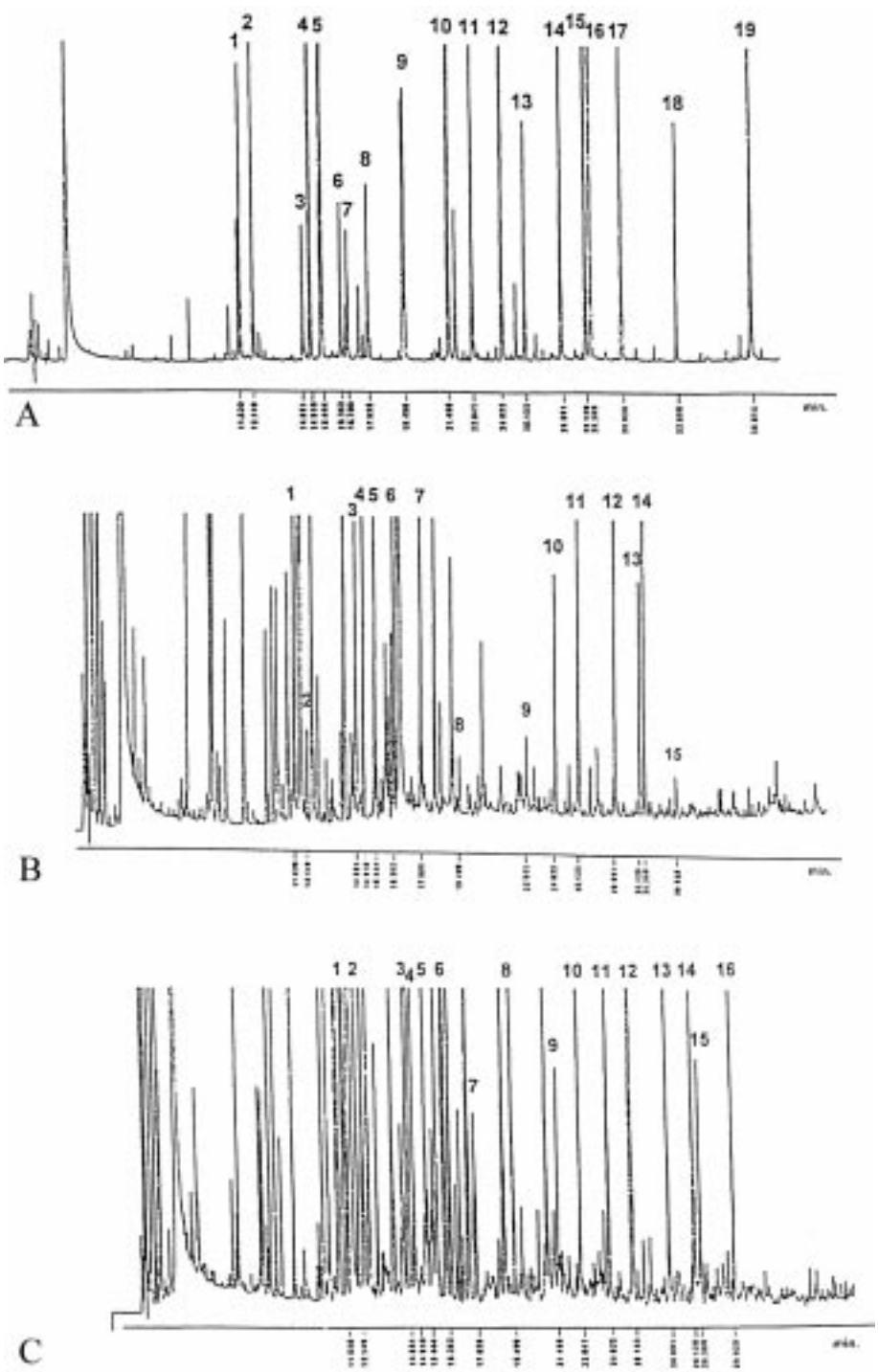

Figure 2. GLC trace of a standard mixture of L-aminoacids by ECD detection, Ala (1), Gly (2), Val (3), Thr (4), Ser (5), Leu (6), Ile (7), Internal Standard (IS) Nor-leu (8), Pro (9), OH-Pro (10), Met (11), Asx (12), Phe (13), Glx (14), Lys (15), Tyr (16), Arg (17), Try (18), Cys (19); 2B: GLC trace of the aminoacids of the yeast cell sample used as control, obtained by ECD detection, Ala (1),Gly (2), Val (3), Thr (4), Ser (5), Leu (6), IS (7), Pro (8), Met (9), Asx (10), Phe (11), Glx (12), Lys (13), Tyr (14), Arg (15); 2C:GLC trace of the aminoacids of the yeast cell sample treated by copper sulphate, Ala (1), Gly (2), Val (3), Thr (4), Ser (5), Leu (6), IS (7), Pro (8), Oh-Pro (9), Met (10), Asx (11), Phe (12), Glx (13), Lys (14), Tyr (15), Arg (16).

Table 4. Aminoacids content (mg/mg dry weight) in yeast cells treated or not with copper sulphate. The values are the means ± Standard Deviation (SD) of three independent determinations.

Aminoacid	TQ		Cu80	
	mean	S.D.	mean	S.D.
Alanine	2.94	± 0.28	1.51	± 0.41
Glycine	0.41	± 0.13	0.67	± 0.08
Valine	1.76	± 0.29	2.76	± 0.23
Threonine	1.37	± 0.42	1.42	± 0.32
Serine	0.94	± 0.42	1.78	± 0.09
Leucine	0.81	± 0.22	1.44	± 0.15
Proline	0.29	± 0.09	0.48	± 0.09
Hydroxi–proline	0.08	± 0.02	0.53	± 0.05
Metionine	0.48	± 0.21	1.09	± 0.04
ASX*	0.64	± 0.08	1.39	± 0.30
Phenilalanine	0.72	± 0.26	3.00	± 0.27
GLX*	5.38	± 1.74	4.67	± 0.17
Lysine	0.82	± 0.38	1.70	± 0.32
Tyrosine	0.54	± 0.06	1.54	± 0.37
Arginine	1.31	± 0.43	1.24	± 0.05

*ASX and GLX indicates, respectively the sum of Asparagine + Aspartic acid and of Glutamine + Glutamic acid, as after derivarisation, they gave the same structure.

Reduced glutathion was indicated as the primary ligand for copper in mammalian cells before the metal transfer to metallothionein and SOD (Freedman *et al.*, 1989). The redox pair involving reduced and oxidized glutathion was also proposed to be essential to maintain copper in the reduced state to bound to apometallothionein (Germann & Lerch 1987). Our results pointing to an increased glutathion level in Cu80 *Candida* cells are, therefore, in line with the hypothesys of an important involvement of this compound in copper resistance. In addition, GSH may be important also with respect to the protection against the products of Fenton-type reaction involving the hydrogen peroxide produced by SOD as scavenger of hydroxyl radicals.

The production of glutathion and metallothionein are linked to the availability of cysteine in the aminoacid pool. This, in turn is expected to be related to the uptake of sulphate from the medium. It is worthnoting that the amount of glutathion found in the cytosolic fraction is maximal when the metal is administered as copper sulphate as compared to copper chloride (Table 2). This result is in acord with previous finding of other authors (Lerch 1980) who showed a dependence of the cellular copper uptake and copper incorporation in metallothionein *versus* the concentration of sulphate in the medium.

The lack of higly statistically significant effects of copper treatment on the catalase levels demonstrates that may not be a coupled behaviour of the levels of this enzyme with that of SOD, as previously found in the case of some *Saccharomyces cerevisiae* strains (Romandini *et al.* 1992). This finding is in apparent contrast with the expected involvement of catalase as a scavenging enzyme for the excess hydrogen peroxide produced by the increased SOD activity elicited by copper treatment (Fridovich 1989; McCord *et al.* 1971). The coupling between SOD and catalase activities is expected to be an important requirement under treatments with a redox active metal ions like copper because the increased citosolic copper levels are expected to increase the probability of $OH^{\cdot}$ radicals formation from H_2O_2 through Fenton chemistry. An enzymatic way to remove hydrogen peroxide, alternative to its dismutation by catalse, is the glutathione peroxidation. Accordingly, reduced glutathion is oxidized in a reaction coupled with reduction of hydrogen peroxide to water. Thus, the increase of total glutathion levels in copper treated cells may have not only the effect of providing the ligand (reduced glutathion) for copper, alternative to metallothionein, but it may support the stimulation of such peroxidase activity as well. Although Fahey & Sundquist (1991) believe the presence of this enzyme in fungi still questionable, Galiazzo *et al.* (1988) have evidences for glutathion peroxidase activity in copper treated yeast. The efficiency of the *in vitro* hydrogen peroxide removal exhibited by our cell extracts of copper treated cells is higher than that expected only on the basis of the azide-sensitive catalase activity and it can be assigned to peroxidase activity. Thus, an assay for hydrogen peroxide removal in the presence of β-mercaptoimidazole to selectively inhibit peroxidase yields an activity attributable to peroxidase activity. It is, therefore, likely that peroxidase activity is indeed present and it increases significantly upon copper treatment. Thus, although we have no direct evidence of specific glutathion peroxidase activity, in view of the increase of total glutathion levels we are tempting to propose that glutathion peroxidation may represent an efficient way of hydrogen peroxide removal. Accordingly, our results show an increase both of total glutathion levels and of peroxidase activity upon copper treatment.

The analysis of the enzyme activities related to the metabolic pathway of carbohydrates has revealed that enolase and triosephosphate isomerase activities significantly decrease upon copper treatment. This ob-

servation could be correlated with the lower growth rate of the microorganism in the presence of copper, depending on an impairement of the energetic pathway.

In the last 30 years extensive developments have been made in the separation and analysis of AA in biological samples by gas chromatographic (GC) analysis. The use of GC for the determination of AA is attractive because it is a sensitive method for their quantitative determination (Weinstein 1966; Husek & Macek 1975; Niederweiser 1975). Furthermore, the use of capillary columns becomes very important for their high speed and resolution capability and, at the end, the availability of chiral phases admit the evaluation of D-isomers.

The analysis of AA from biological samples, now represented by cells, is hampered by two problems: first, raising a sufficient quantity of sample to analyse (the number of yeast cells obtained from a culture is a critical point) and second, the purification of the sample from the components of the utilized medium. Thus, the complete procedure for determining AA in biological and physiological fluids will involve the separation of interfering substances from AA followed by chromatographic resolution and quantitation of the latter. Since free AA are not enough volatile for gas chromatography, due to their high polarity, it is necessary to prepare volatile derivatives (Bayer *et al.* 1987) such as the N-heptafluorobuthyle derivatives obtained by us. This improves AA volatility, but, because of the addition of at least seven atoms of fluoride to their molecules, they may be detected not only with a flame ionisation detector (FID) but with an electron capture detector (ECD) as well.

The ECD detector is very sensitive to halogens and this improves the detection limit of the derivatized AA; De Ming (1989) successfully used this fact to study the presence of AA in geologycal enviroments. In the present case yeast cells grown in 20 ml broth culture were enough to obtain a detectable signal by ECD, while when FID was used, not less than 100 ml were mandatory. This is an important result for the microbiological application because a large amount of culture medium could determine a dispersion and consequently a loss in cell number during their collection. Furthermore, a lowering of the amount of broth culture could make easier the concentration of the samples and their subsequent analyses.

Statistically significant increases in the content of several aminoacids are found. In some istances the effects are very remarkable and imply a two- to ten-fold increase of amino acid content. Although more work is needed to understand the correlation between the copper treatment and the stimulation of amino acid sinthesys it is suggested that the increase of aminoacid levels may support the synthesys of the key proteins involved in the detoxification system against the excess copper. Another hypothesys that it could be put forward involves the increase of amino acids as an accumulation of potential bifunctional ligating groups, able to bind copper inside the cell, that could limit the stress due to the metal presence in the broth culture.

References

Adams RF. 1974 Determination of aminoacids profiles in biological samples by gas-chromatography. *J Chromatogr* **95**, 189–212.

Anderson ME. 1985 Determination of glutathione and glutathione disulfide in biological samples. In: Meister A, ed. *Methods in enzymology*, New York: Academic Press: **13**, 548–555.

Bayer E, Frank H, Gerhardt J, Nicholson G. 1987 Capillary gas chromatographic analysis of amino acids by enentiomer labeling. *J Assoc Off Anal Chem* **70** (2), 234–240.

Beutler E. 1984 A Manual of Biochemical methods in *Red Cell Methabolism*. New York: Grune and Stratton Inc.: 3rd ed.

Bilinski T, Litwinska J. 1987 On the ideas alternative to the theory of superoxide-mediated oxygen toxicity. *Bull Polish Acad Sci* **35**, 25–31.

Butt TR, Ecker DJ. 1987 Yeast metallothionein and applications in biotechnology. *Microbiol Rev* **51** (3), 351–364.

Cousins RJ. 1985 Absorption, transport, and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin. *Physiol Rev* **65**, 238–309.

De Ming L. 1989 Capillary gas chromatographic analysis of amino acids in geological environments with electron capture detection. *J High Resol Chrom* **12**, 239–243.

Etcheverry T, Forrester W, Hitzeman R. 1986 Regulation of chelatin promoter during the expression of human serum albumin or yeast phosphoglycerate kinase in yeast. *Biotechnology* **4**, 726–730.

Fahey RC, Sundquist AR. 1991 Evolution of glutathione methabolism. *Adv Enzymol Relat Areas Mol Biol* **64**, 1–53.

Freedman JH, Ciriolo MR, Peisach J. 1989 The role of glutathion in copper metabolism and toxicity. *J Biol Chem* **264**, 5598–5605.

Fridovich I. 1989 Superoxide dismutases. An adaptation to a paramagnetic gas. *J Biol Chem* **264**, 7761–7764.

Galiazzo F, Schiesser A, Rotilio G. 1988 Oxygen independent induction of enzyme activities related to oxygen metabolism in yeast by copper. *Biochem Biophys Acta* **965**, 46–51.

Germann UA, Lerch K. 1987 Copper accumulation in the cell-wall-deficient slime variant of *Neurospora crassa*. Comparison with a wild type strain. *Biochem J* **245**, 3678–3670.

Husek P, Macek K. 1975 Gas chromatography of amino acids.*J Chromatography* **113**, 139–230.

Karin M, Najarain R, Haslinger A, Valenzuela P, Welch J, Fogel S. 1984 Primary structure and transcription of an amplified genetic locus: the CUP1 locus of yeast. *Proc Natl Acad Sci* **81**, 337–341.

Lerch K. 1980 Copper metallothionein, a copper-binding protein from *Neurospora crassa*. *Nature* **284**, 3678–3700.

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. 1951 Protein measurement with the Folin phenol reagent *J Biol Chem* **193**, 265–275.

Mc Cord JM, Keele BB, Fridovich I. 1971 An enzyme based theory of obligate anaerobiosis, the physiological functions of superoxide dismutase. *Proc Natl Acad Sci (USA)* **68**, 1024–1027.

Mac Kenzie SL, Tenaschuk D. 1974 Gas-liquid chromatography of N-heptafluorobutyryl isobutyl esters of aminoacids. *J Chromatography* **97**, 19–24.

Michelson AM, Puget K, Durosay P, Nonneau JC. 1977 Clinical aspects of the dosage of erytrocuprein. In: Michelson AM, Mc Cord JM, Fridovich I eds. *Superoxide and superoxide dismutase*. London: Academic Press 467–499.

Niederweiser A. 1975 Chromatography, E. Heftmann Ed. New York: Van Nostrand Reinhold. Vol. II, 393.

Puget N, Michelson AM. 1974 Iron containing superoxide dismutase from luminous bacteria. *Biochimie* **56**, 346–356

Puget N, Michelson AM, Avrameas S. 1977 Light emission techniques for the microestimation of femtogram levels of peroxidase. *Anal. Biochem.* **79**, 447–456

Romandini P, Tallandini L, Beltramini M, Salvato B, Manzano M, de Bertoldi M, Rocco GP. 1992 Effects of copper and cadmium on growth, superoxide dismutase and catalase activities in different yeast strains. *Comp Biochem Physiol* **103** (C), 255–262.

Rose AH, Veazey FJ. 1988 Membranes and lipids of yeasts. In: Campbell I, Duffus JH. eds. *Yeast a practical approach*. Oxford: IRL Press. 255–275.

Sarais I, Manzano M, de Bertoldi M, Romandini P, Beltramini M, Salvato B, Rocco GP. 1994 Adaptation of a *Saccharomyces cerevisiae* strain to high copper concentration. *Biometals* **7**, 221–226.

Steinebach OM, Wolterbeek HT. 1994 Role of cytosolic copper, metallothionein and glutathione in copper toxicity in rat hepatoma tissue-culture cells. *Toxicology* **92**, 75–90.

Stadtman ES. 1990 Metal ion-catalyzed oxidation of proteins: biochemical mechanisms and biological consequences. *Free Radical Biology & Medicine* **9**, 315–325.

Stadtman ER, Oliver CN. 1991 Metal-catalyzed oxidation of protein. *J Biol Chem* **266**, 2005–2008.

Stocchi V, Cardoni P, Piccoli G, Cucchiarini G, De Belli G, Dachà M. 1994 High resolution of multiple forms of rabbit reticulocyte hexokinase type I by hydrophobic interactin chromatography. *J Chromatography* **676**, 51–63.

Thiele D. 1988 ACE1 regulates expression of the *Saccharomyces cerevisiae*. metallothionein gene. *Mol Cell Biol* **8**, 2745–2752.

Tohoyama H, Inouhe M, Joho M, Murayame T. 1995 Production of metallothionein in copper and cadmium resistant strain of *Saccharomyces cerevisiae*. *J Industr Microbiol* **14**, 126–131.

Tran LT, Miki T, Kamakura M, Izawa S, Tsujimoto Y, Miyabe S, Inoue Y, Kimura A. 1995 Oxidative stress-response in Yeast - Introduction of glucose-6-phosphate-dehydrogenase by lipid hydroperoxide in *Hansenula mrakii*. *J Ferm Bioe* **80**, 606–609.

Viarengo A, Nott JA. 1993 Mechanisms of heavy metal cation homeostasis in marine invertebrates. *Comp Biochem Physiol* **104**(C), 355–372.

Weinstein B. 1966 In: *Methods of Biochemical Analysis*, New York: John Wiley & sons: **14**, 203.

Wilkinson L. 1990 *Systat, The system for statistic*. Evanston, Inc.